PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF FRUIT EXOCARP OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL) MUTANTS TO NATURAL PHOTOOXIDATIVE CONDITIONS

By

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of Carolina A. Torres del Campo find it satisfactory and recommend that it be accepted.

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Chair

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PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF FRUIT EXOCARP OF TOMATO (*LYCOPERSICON ESCULENTUM* Mill) MUTANTS TO NATURAL PHOTOOXIDATIVE CONDITIONS

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Photooxidative damage in fruit occurs under high temperature and solar radiation conditions, and results in sunscald with surface discoloration and bleaching. Using a system that permitted the imposition of photooxidative stress under natural solar radiation, we evaluated symptoms development, photosynthetic efficiency, pigment and flavonoid composition, and antioxidant metabolites and enzymes in exocarp of immature green tomato (*Lycopersicon esculentum* Mill) fruit. Exposed or covered sections of detached fruit of mutants (*anthocyanin absent*, *β-carotene*, *delta*, and *high pigment-1*) with attenuated pigment and/or antioxidant metabolite levels, and their nearly isogenic parents ('Ailsa Craig' or 'Rutgers'), were subjected to five hours of high solar irradiance, either in the presence or absence of ultraviolet (UV) radiation. Photooxidative stress on detached tomato fruit reproduced sunscald symptoms on attached fruit. Both high temperature and solar irradiance caused fruit surface discoloration with

faster degradation of chlorophyll (Chl) than carotenoids (Car), leading to an increase in the Car/Chl ratio. Bleaching of the fruit surface was mostly caused by solar irradiance, whereas high temperature was responsible for most inactivation of photosynthesis.

Quercetin and kaempferol concentrations increased rapidly upon exposure to sunlight, but not to natural UV radiation, suggesting rapid photo-protection by these flavonoids in response to visible light. Ascorbate and glutathione concentrations decreased as duration of sun exposure increased, but activities of antioxidant enzymes (SOD, APX, DHAR, MDHAR, GR), as well as MDHAR and GR protein levels, increased to maintain the ascorbate and glutathione pools in its their reduced forms. These responses prevented the accumulation of lipid peroxidation products and possibly protein oxidation and DNA damage, suggesting that damage to these macromolecules might not be detectable until cellular antioxidant systems are unable to cope with increasing ROS flux during photooxidative stress.

Fruit exocarp from *high pigment-1* had higher Chl and Car levels than the other genotypes, and more kaempferol, quercetin, and naringenin than ‘Ailsa Craig’. Nevertheless, but it suffered photoinhibition to the same extend as its parent. This led to the conclusion that the higher ascorbate pool, APX and GR activities, and flavonoid concentrations in *high pigment-1* fruit exocarp allowed it to better cope with the imposed photooxidative stress.

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Dedication

I cannot express how blessed I feel for having you…Ricardo. Without you or our beautiful son Bruno this would have never happen. I love you.

CHAPTER 1

PHOTOOXIDATIVE INJURY OF FLESHY FRUIT

REVIEW

I. Introduction

Solar radiation drives many vital light-dependent processes in plants, such as photosynthesis and photomorphogenesis. However, when green tissues are incapable of coping with excessive energy, solar radiation can be detrimental. Immature fruits can be subjected to light-mediated tissue deterioration known as photooxidative damage. Photodynamic injury of heated tissue is a type of photooxidative damage that occurs under intense sunlight and elevated temperature conditions. In fruit, it is also commonly called sunscald (Barber and Sharpe, 1971; Bergh *et al.*, 1980; Retig and Kedar, 1967; Walker, 1957), and sometimes "sunburn". Symptoms of sunscald can appear on fruit acclimated to high solar radiation, such as those that have been continuously exposed to the sun (Moore and Rogers, 1943), or on non-acclimated fruit that are suddenly exposed, such as occurs after pruning, natural or artificial spreading of branches, or loss of foliage from diseases (Ramsey *et al.*, 1952; Retig and Kedar, 1967).

While sunscald symptoms may vary, three general types were defined by Barber and Sharpe (1971): 1) heat-injury sunscald (HIS) with a cooked appearance; 2) ultra-violet radiation sunscald (UVS), present in fruit growing at high elevations; and 3) photodynamic sunscald of heated tissues (PSHT), caused by chemical lesions of

reactions of reactive oxygen species (ROS) induced by high temperature in photosensitive, pigmented cells that have absorbed excess visible solar radiation.

The observed symptoms may vary among species and even among cultivars within species. However, they always involve discoloration (yellowing, browning, reddening, blackening) or bleaching of the affected area due to degradation of chlorophyll. Sometimes these lesions become flattened or sunken and may be followed by tissue desiccation (Ramsey *et al*., 1952; Barber and Sharpe, 1971; Brooks and Fisher, 1926). In the case of tomatoes (*Lycopersicon esculentum* Mill.), lack of lycopene on affected areas is due to reduced synthesis of this carotenoid at temperatures above 30°C, which is sometimes called “irregular ripening” (Ramsey *et al*., 1952; Retig and Kedar, 1967; Tomes *et al*., 1956). This symptom (also called “white spot”) is usually surrounded by a yellow halo (Rabinowitch *et al*., 1974). In peaches, severe sunscald damage can cause skin (exocarp) rupture and flesh (mesocarp) exposure (Moore and Rogers, 1943). There is evidence showing that sunscald damage is directly correlated with increased electrolyte leakage indicating loss of cell integrity (Prohens *et al*., 2004)

Another type of solar radiation damage in raspberries, referred to as “white drupelet disorder”, occurs when high solar radiation causes inhibition of anthocyanin synthesis in individual drupelets of the fruit (Renquist *et al*., 1989). Although this symptom remains until fruit are fully ripe, typical tissue browning or desiccation does not occur as in other species.

An additional problem with sunscald-injured fruit is that affected tissue can become an easy target for fungal infection, softening, or other post-harvest disorders,

such as superficial scald in apples (Barber and Sharpe, 1971; Fallik *et al*., 1997; Gatti *et al*., 1986; Moore and Rogers, 1943). Alternaria rot (*Alternaria* sp.) is a disease that is normally found following sunscald damage in tomatoes and peppers (Ramsey *et al*., 1952). In tomatoes, sunscald inhibits normal ripening by delaying softening, blocking protopectin conversion, reducing lycopene accumulation, suppressing respiration, and initially inhibiting autocatalytic ethylene production (Adegoroye and Jolliffe, 1987; Adegoroye *et al*., 1989). There is also evidence that the development of "bronzing" on strawberry fruit might be a consequence of an early sunscald lesion that leads to changes in subepidermal parenchyma cell and abnormal pigmentation and phenolics accumulation in the affected area (Polito *et al*., 2002).

Sunscald damage can be severe enough to cause economic losses to fruit of several crops, including peach (Moore and Rogers, 1943), tomato (Moore and Rogers, 1943; Rabinowitch *et al*., 1974; Ramsey *et al*., 1952; Retig and Kedar, 1967), banana (Wade *et al*., 1993), melon (Lipton, 1977), raspberry (Renquist *et al*., 1989), apple (Brooks and Fisher, 1926; Dooley and Brohier, 1986; Moore and Rogers, 1943; Schrader, 1997; Yuri *et al*., 1996), peppers and cucumbers (Barber and Sharpe, 1971; Fallik *et al*., 1997; Rabinowitch *et al*., 1983, Ramsey *et al*., 1952), and eggplants (Roberts and Anderson, 1994). In apples, sunscald constitutes the most common cosmetic defect in areas where high solar radiation and temperatures occur during the growing season, such as in semi-arid regions of Washington State (United State of America), Australia, South Africa, Israel, and the Central Valley in Chile. Sunscald damage can reduce fruit grade or cause cullage so that fruit cannot be packed for the fresh market (Yuri *et al*., 1996). 'Fuji' apple orchards in Chile have shown as high as

40% cullage from sunscald in the orchard. In the packinghouse, apples eliminated due to sunscald can be as much as 21% of the total discarded. This amount of cullage has resulted in more than a 28 million US$ loss for the apple export industry in Chile (Yuri *et al.*, 1996). For Washington State apple growers, the economic loss from fruit affected by sunscald has been estimated to be about $100 million per year, with the incidence of sunscald becoming more common as plantings of smaller trees on dwarfing rootstocks have become widespread (Schrader, 1997).

II. Factors affecting photooxidative fruit injury

Species or cultivar susceptibility to sunscald is mainly determined by environmental factors that include: 1) interception of solar radiation, 2) solar absorptivity, and 3) energy dissipation (reflectivity, long wave emittance, transpiration, convective heat loss, latent heat of vaporization) (Clendenning, 1941; Levitt, 1951, 1958); and physiological factors that include 1) developmental stage; 2) specific photo-stability due to differences in the carotenoid-chlorophyll complex (Barber and Sharpe, 1971), 3) ultraviolet (UV) radiation tolerance influenced by the degree of pigmentation (Tanada and Hendricks, 1953), and 4) degree of adaptation and/or acclimation, especially to high temperatures (Barber and Sharpe, 1971).

A. Environmental Factors

1. Temperature

Solar irradiance results in high tissue temperature on the exposed surface of the fruit. Surface temperature is strongly affected by the intensity of irradiance and wind,

which can dramatically reduce fruit surface temperatures (Bergh *et al*., 1980; Retig and Kedar, 1967). In comparative studies of shaded and exposed apples, temperature differences from 5 to 18°C (Yuri *et al*., 1998) and 5.6 to 8.9°C (Brooks and Fisher, 1926) have been reported. In addition, Brooks and Fisher (1926) found that the temperature of the exposed surface of an apple was 6.7 to 8.3°C higher than air temperature. Severe sunscald symptoms in apples have been observed when surface temperatures of exposed fruit exceeded 50°C, and when air temperatures exceeded 36°C (Bergh *et al*., 1980). Prohens *et al*. (2004) reported that exposed pepino (*Solanum muricatum*) fruit was 12.5°C higher that air temperature. They also concluded that temperatures above 42°C were necessary to induce severe sunscald symptoms.

Tissue water content has been found to be important in the process of fruit heating and ultimately sunscald because water absorbs infrared (IR) radiation. IR radiation constitutes about 60% of the energy of solar radiation (Campbell and Norman, 1998).

Barber and Sharp (1971) found that smaller fruit were more resistant to sunscald than larger fruit from the same species and cultivar because smaller fruit have a higher surface area:volume ratio, and so, a higher efficiency for convective heat loss that leads to lower surface temperatures. The degree of damage obtained by heat treatment has been shown to depend on pericarp temperature and treatment duration. Rabinowitch *et al*. (1974) showed that threshold temperatures for sunscald of tomatoes were between 39.1°C and 40.8°C. They also found that light was required to induce sunscald symptoms. Thus, heat plus light, imposed during both the “induction” period, where fruit

were exposed to 45°C for 18 h, and the "incubation" period, where fruit were kept at 25°C for 60 h, were the most effective treatments, resulting in sunscald of all fruits.

2. Solar Radiation

In addition to heat, light is an essential component in the development of sunscald. This has been clearly shown in tomatoes, peppers, and cucumbers by Rabinowitch *et al*. (1974, 1983). In tomatoes, when high temperature was applied under controlled conditions, but without light, the symptoms that developed were mainly those of heat damage, with tissue appearing "boiled", that is, water soaked, soft, but mostly green with a slight brown discoloration. Typical sunscald symptoms, that is, browning, bleaching, or necrosis did not appear under field conditions (Rabinowitch *et al*., 1986).

Ultraviolet light (UV), especially UV-B (280-320 nm) has been considered a contributing factor to sunscald. A study conducted by Renquist *et al*. (1989) showed that symptoms of "white drupelet disorder" in raspberries were obtained when fruit were exposed artificially to a combination of 42°C or higher temperature and 4-7 h of UV-B light with fruit damage proportional to time of exposure to UV-B. Fruit damage was reduced when UV filters were used, that is, cellulose acetate (blocks UV-C) and Mylar® (blocks most UV). The effects of UV radiation on sunscald has not been clearly shown in other fruits, such as apples, although there have been some investigations. Bastías (1998) reported UV radiation injury to 'Granny Smith' apples. In these experiments, treatment with high temperatures (40-42°C skin surface) and UV-A plus UV-B light under controlled conditions caused heat damage symptoms (but not sunscald symptoms) to the exposed side of whole fruits. Experiments conducted by Schrader

(1997), also with apples, indicated that UV-B radiation alone did not cause sunscald, but it did exacerbate heat damage.

B. Plant-Associated Factors

1. Acclimation

Exposure and subsequent acclimation to high temperatures has been reported to increase tolerance to sunscald. Fruit grown at or exposed to elevated temperatures prior to exposure to high light and high temperature were more tolerant. Fruit acclimation to heat injury through pre-treatment of detached fruit to sub-damaging temperatures has been shown for avocado (Schroeder, 1963), tomato (Retig and Kedar, 1967; Retig *et al.*, 1974), and cucumber and pepper (Rabinowitch *et al.*, 1986). In all cases, acquired tolerance to sunscald was achieved with short exposure to high temperatures prior to insolation of the fruit. Under these circumstances photodynamic processes did not occur. These treatments, therefore, only temporarily induced resistance. In a tomato study, Retig and Kedar (1967) found that the acclimation process occurred both in the light (field) and in the dark (inside a black bag), so they associated the phenomenon to heat and not other components of insolation. Kedar *et al.* (1975) also came to the same conclusion in another experiment with tomatoes.

Brooks and Fisher (1926) reported fruit acclimation to sunscald under natural conditions in apples. They compared different growing climates in different sites in the USA. Their findings indicated that the temperature regime had a great influence on heat tolerance. If fruit acclimation to heat occurred gradually, increased tolerance to higher temperatures occurred. They also documented that partially shaded fruit lacked this

tolerance, and therefore, shaded fruit developed sunscald easily when suddenly exposed to sunlight.

2. Pigmentation

Fruit pigmentation, due both to the type and concentration of pigments, has a direct influence on surface temperature (Rabinowitch *et al.*, 1983; Retig and Kedar, 1967). A study by Barber and Sharpe (1971) showed that immature green *Capsicum annuum* L. 'California Wonder' fruit reached 9°C higher skin temperature than the skin of ivory-yellow 'College Gold' fruit at maximum air temperature. They also found that in 'California Wonder' the incidence of photodynamic sunscald injury varied from 0 to 12%, which was inversely proportional to the leaf/fruit ratio, whereas in 'College Gold' the incidence of sunscald was only 0 to 2%, and independent of leaf/fruit ratio. Barber and Sharpe (1971) found a similar temperature effect in *Cucurbita pepo* L. fruits in a comparison of dark-green fruit of 'Bush Green' and light-green (immature) and orange (mature) fruits of 'Bush White'. The authors explain these susceptibility differences between cultivars of the same species by differences in total energy absorbed by the surface of the fruit. When these differences were analyzed it was determined that the IR energy reflected by *Cucurbita* fruit was greater than that by *Capsicum* fruit, apparently because of differences in distribution and size of intercellular spaces in the outer region of the fruit.

3. Maturity

Sunscald susceptibility varies depending upon the stage of fruit maturity. Mature-green tomatoes (Adegoroye and Jolliffe, 1983; Retig and Kedar, 1967; Retig *et al*., 1974), peppers (*Capsicum annum*), and cucumbers (*Cucumis sativus*) (Rabinowitch *et al*., 1983; Rabinowitch and Sklan, 1981) showed the highest sunscald susceptibility. Ripe red tomatoes, red peppers, and yellow-ripe cucumbers were the most tolerant. Reduced chlorophyll levels in mature fruit may explain these observations, because less photooxidative damage at high temperatures is likely to occur (Barber and Sharpe, 1971; Rabinowitch *et al*., 1974). However, there is evidence indicating that mature red fruit, which generally do not show visible sunscald symptoms, have some level of damage, masked afterwards by secondary fungal infection (Barber and Sharpe, 1971). In addition, Retig and Kedar (1967) showed that red fruit had higher surface temperatures than mature green fruit, even though red fruit were highly resistant to sunscald.

III. Physiological and Biochemical Factors Associated with Photooxidation

A. Oxidative Processes

Sunscald injury, as well as many other environmental, physical, and xenobiotic stresses, involves mainly photooxidative reactions (visible as chlorophyll bleaching), which are produced by an increase in the flux of oxyradicals or ROS in cells (Foyer *et al*., 1994). However, this has never been documented in photooxidative fruit damage. ROS are toxic compounds generated by incomplete reduction of molecular oxygen (O_2) or incomplete oxidation of water by mitochondrial or chloroplastic electron transfer

chains. ROS include: 1) superoxide anion ($O_2^{\cdot -}$), the primary product of photoreduction of O_2 at photosystem I (PSI) (Asada *et al.*, 1974); 2) hydrogen peroxide (H_2O_2); 3) hydroxyl radical ($OH^{\cdot}$), generated by the reaction of H_2O_2 with metal reductants Fe(II) and Cu(II); 4) and singlet oxygen ($^1O_2$) (Foyer, 1984; Halliwell, 1995; McKersie and Leshem, 1994; Salin, 1988a). Under normal photosynthetic photon flux densities (PPFD), the generation rate of ROS is low, so the antioxidant systems in the cell are capable of efficiently scavenging them. When high light plus high temperatures are present, which could lead to a sunscald event, oxyradical formation is greatly increased, especially in processes involving photodynamic reactions. These reactions occur in green tissue and involve three components: a photosensitizer (e.g. chlorophyll, riboflavin, griseofulvin, porphyrins), light energy, and oxygen (Blum, 1941; Chaudière and Ferrari-Liou, 1999). When exposed to light energy (at a certain wavelength depending on the compound), the photosensitizer becomes energized to an excited triplet state, and can then transfer this energy to adjacent oxygen molecules, creating highly reactive singlet oxygen. Under excess energy conditions, caused either by high PPFD and/or reduced light utilization capacity, as occurs under during environmental stresses (e.g. high temperature), excited triplet-state chlorophyll ($^3Chl^*$) molecules can transfer their energy to dioxygen molecules (O_2, normal triplet ground state) forming $^1O_2$ (Foyer, 1993), which can damage the photosynthetic apparatus. Thereby, photosynthesis is inhibited, in a process referred to as "photoinhibition".

Recent research conducted by Hideg *et al*. (2002) has suggested that leaves exposed to high PPFD suffered photoinhibition mainly due to excess production of $^1O_2$, and less to $O_2^{\cdot -}$ production. The opposite was found to be true when UV radiation stress

(UV-A plus UV-B) was given, where 90% loss of photosynthetic activity was caused by accumulation of $O_2^{\cdot -}$ and not $^1O_2$. Damage to the photosynthetic apparatus (i.e. PSII redox components and water-oxidizing complex) by UV radiation was earlier reported by Vass *et al*. (1996) and Bornman (1989).

Photoinhibition of photosynthesis occurs prior to photooxidative damage. Photoinhibition may be due to a reduction of photosynthetic energy conversion caused by either an increase in thermal energy dissipation, which is actually a photoprotective mechanism via the xanthophyll cycle, or by damage to PSII (e.g. D1/32-kDa herbicide-binding/Q_B protein degradation) (Landgraf *et al*., 1997), which is the main target of photoinhibition under excess light rather than PSI (Asada, 1999; Demmig-Adams and Adams III, 1992a). Photoinhibition *in vivo* occurs when the rate of D1 protein turnover, including protein degradation, de novo-synthesis and re-assembly to reactivate PSII complexes is surpassed (Aro *et al*., 1993a, 1993b).

The occurrence of photoinhibition of photosynthesis can be assessed by measuring a decrease in the ratio of maximum variable to maximum total fluorescence (Fv/Fm; measured after a dark-period following high light exposure), which indicates the maximal potential photosynthetic efficiency (i.e. intrinsic efficiency of PSII). The feasibility of this measurement is based on the fact that dark-adapted tissue is totally accessible to photochemical reactions and non-photochemical dissipative pathways (e.g. via xanthophylls cycle) are minimal (Krause and Weis, 1991).

According to a study of several species of fleshy fruits by Smillie (1992), both fruit and leaves of a species had comparable photosynthetic capacities in spite of differences in chlorophyll contents. However, studies of tomato by Hetherington *et al*.

(1998) and Smillie *et al*. (1999) showed that the efficiency of PSII decreased more drastically in fruits than in leaves with increasing PPFD. Hence, non-photochemical quenching (NPQ) increased faster in fruit than in leaves with increasing PPFD. This indicates that green fruit, although potentially contributing to fruit growth via carbon assimilation, would have a lower capacity to utilize solar energy, and therefore, would suffer photoinhibition at lower PPFD than leaves.

Changes in chlorophyll fluorescence parameters, especially in leaves, also have been positively correlated with many biotic and abiotic stresses, such as heat, freezing, drought, and pathogen attack (Van Kooten *et al*., 1990; Somersalo and Krause, 1990). In fruit this technique has been used to correlate chlorophyll content, or photosynthetic capacity, to ripening (Jacobi *et al*.,1998; Blackbourn *et al*., 1990). For instance, chlorophyll fluorescence has been proposed as a non-destructive tool to predict maturity in mango (*Mangifera indica* Linn) fruit. As fruit maturity or senescence increased, measured as changes in skin fruit color or loss of chlorophyll content, Fv and Fo (basal fluorescence) decreased, which indicates lower PSII activity (Jacobi *et al*.,1998).

Ludlow and Björkman (1984) described two types of photoinhibitory events occurring at different temperature ranges in Siratro (*Macroptilium atropurpureum* Moc. & Sessé ex DC.) leaves. The first event occurred at temperatures ranging between 31 and 42°C when plants were submitted to high light and some other stress, such as water stress. They referred to this event as “high-temperature-exacerbated photoinhibition”. In this case, full or partial recovery occurred if temperatures or light levels decreased. In general, high light, water stress, and high temperatures were additive. The second type of photoinhibition occurred at temperatures over 42°C in the presence of high light, and

was called "high-temperature-induced photoinhibition". This type of photoinhibition was either reversible when the temperature was between 42 and 48°C or irreversible when the temperature was over 48°C, regardless of the presence of light or other stresses. Irreversibility depended on leaf temperature, time of exposure, recovery conditions, and degree of temperature acclimation by the plant. Armond *et al*. (1980) working with isolated thylakoid membranes from *Nerium* plants grown under different temperature regimes reported that heat stress induced not only a functional but also a physical dissociation of PSII core complex from the chlorophyll a/b light-harvesting complex, thus decreasing the energy transfer efficiency between pigment complexes. This event was delayed in plants grown in higher temperature regimes, probably because of acclimation.

Many of these events that occur in leaves may also occur in fruit when they are exposed to high solar radiation and temperatures during development. In apples, for example, sunscald symptoms are particularly noticeable after high temperature events that occur over successive days, which may be due to a sudden increase in ROS due to either high-temperature-exacerbated or high-temperature-induced photoinhibition as described by Ludlow and Björkman (1984). Similarly, water-stressed apple trees are usually more severely affected by sunscald than are well-watered trees located nearby.

B. Antioxidants

In chloroplasts and throughout the cell, ROS are detoxified by antioxidants. A broad definition of antioxidant is "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or

prevents oxidation of that substrate" (Halliwell, 1995). Antioxidants can act as chemical traps of ROS or physical quenchers of excited species, such as $^1O_2$ or $^3Chl^*$ (Chaudière and Ferrari-Liou, 1999).

Antioxidants include metabolites, such as ascorbic acid (AsA) and reduced glutathione (GSH), enzymes, such as superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6), and other secondary compounds, such as β-carotene, α-tocopherol, anthocyanins, and phenolics (McKersie, and Leshem, 1994; Salin, 1988a). Among antioxidant enzymes are those involved in the Mehler–Ascorbate–Peroxidase cycle (part of the water-water cycle) such as SOD, ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), and dehydro- and monodehydro-ascorbate reductase (DHAR; EC 1.8.5.1 and MDHAR; EC 1.6.5.4) (Asada, 1999; Foyer and Halliwell, 1976; Foyer *et al*., 1994). The Mehler–Ascorbate–Peroxidase cycle (Fig. 1), as part of the water-water cycle, includes the enzymes responsible for AsA and GSH recycling, keeping these metabolites biologically active as antioxidants. The main physiological functions of the water-water cycle are to: 1) protect antioxidant enzymes (thylakoidal bound and stromal) and PSI complexes (especially [4Fe-4S] clusters) from oxidative damage by ROS (especially photo-produced $O_2^{\cdot -}$ in PSI), 2) supply ATP to maintain CO_2 assimilation, and 3) provide photoprotection by dissipating excess energy using O_2 as electron acceptor (Asada, 1999).

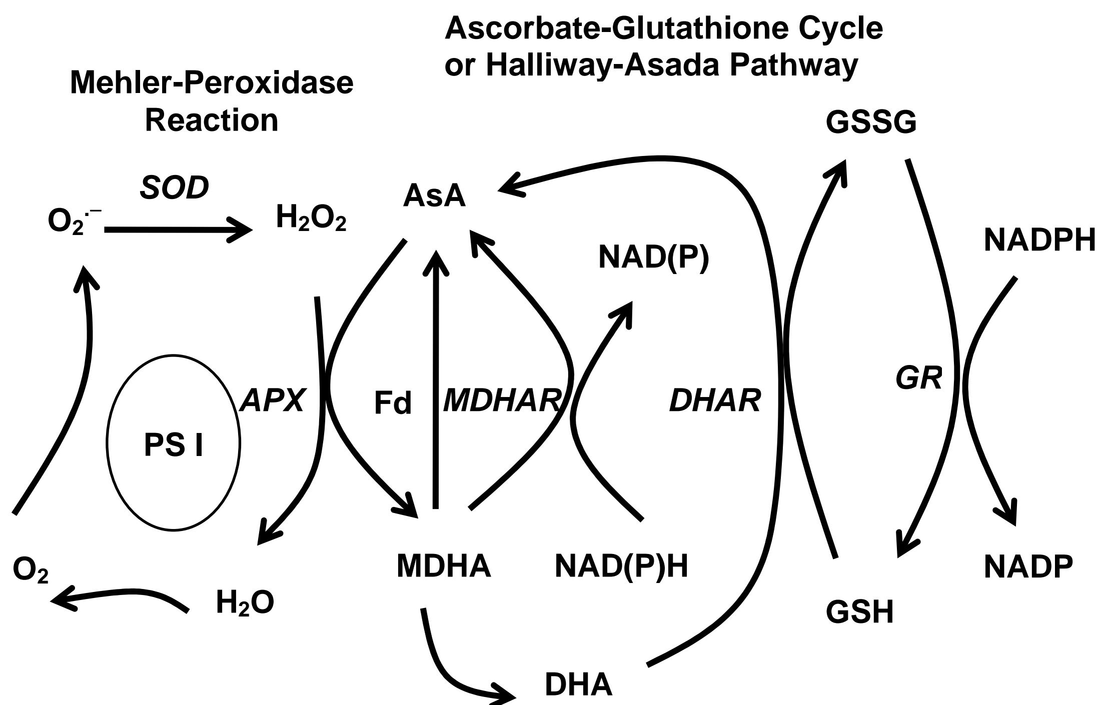

Figure 1. Mehler-Ascorbate–Peroxidase cycle (also called water-water cycle).

PS I: Photosystem I; SOD: superoxide dismutase; APX: ascorbate peroxidase: AsA: ascorbate (ascorbic acid); MDHAR: monodehydroascorbate reductase; Fd: Ferredoxin; MDHA: monodehydroascorbate; DHAR: dehydroascorbate reductase; DHA: dehydroascorbate; GSSH: oxidized glutathione, disulphide bond; GSH: glutathione; GR: glutathione reductase.

All these enzymes and metabolites have been positively correlated with resistance to a number of stresses, such as drought (Gamble and Burke, 1984; Robinson and Bunce, 2000), salinity (Shalata and Tal, 1998), chemicals (Kraus and Fletcher, 1994), heat (Kraus and Fletcher, 1994), chilling temperatures (Schöner *et al.*, 1990; Kingston-Smith *et al.*, 1997), and ozone (Lyons *et al.*, 1999).

Karpinski *et al.* (1997) showed evidence for a mechanism in plants for coping with excess light events, which leads to photooxidative damage that was regulated

partially by the redox-state of the plastoquinone pool. This mechanism of redox regulation rapidly activated the expression of genes, such as *APX1* and *APX2* (nuclear-encoded cytosolic ascorbate peroxidase), that are involved in H_2O_2 scavenging. The redox state of the glutathione pool (GSH/total glutathione) decreased after these high-light events, which indicated the presence of an oxidative burst. The change in the glutathione redox state was also found to be involved in signal transduction, leading to activation of the APX genes.

1. Ascorbic acid and Glutathione

Among hydrophilic scavengers AsA and GSH are the most important. L-Ascorbic acid or vitamin C, besides being an essential nutritional component of the human diet, has an important role in environmental stress resistance, including photooxidation (Conklin *et al*., 1996; Foyer, 1993; Noctor and Foyer, 1998; Smirnoff, 2000), where it acts as a free radical reductant in plant tissues, thereby reducing oxidative damage. The first genetic evidence for this property was provided by studies carried out in an ascorbate-deficient *Arabidopsis thaliana* mutant *vtc1* (vitamin c-1, also called *soz1*), an ozone-sensitive mutant that had only 25-30% of wild-type ascorbate content that is also hypersensitive to SO_2 and UV-B stress (Conklin *et al*., 1996). The *vtc1* mutation is due to a defect in the biosynthetic pathway of ascorbate from D-glucose (Conklin *et al*., 1997), specifically the GDP-mannose pyrophosphorylase enzyme (Conklin *et al*., 1999).

Ascorbic acid is ubiquitous in plants being absent only in dry seeds. It has important functions in other physiological processes in plants, such as growth, differentiation, and metabolism (Foyer, 1993). There is evidence indicating that at least

a portion of AsA in green plants and chlorophytic algae is synthesized in the cytosol as a product of D-glucose-6-phosphate via D-mannose and L-galactose in an eight-step enzymatic pathway called the Smirnoff-Wheeler pathway (Wheeler *et al*., 1998; Smirnoff *et al*., 2001; Smirnoff, 2003). Agius *et al*. (2003) have shown that, at least in strawberry fruit, AsA is also synthesized during ripening from D-galacturonic acid, a component of cell-wall pectins. They discovered evidence for this by isolating and expressing the gene encoding D-galacturonate reductase (*GalUR*), a key enzyme in D-galacturonic acid metabolism to L-ascorbic acid.

Ascorbic acid has been found in chloroplasts, vacuoles, and extracellular compartments of the cell. Between 20 and 40% of the total AsA found in leaf mesophyll cells is localized in chloroplasts (20-300 mM). This organelle, as well as the cytosol of non-photosynthetic cells, contains all the enzymes needed to regenerate reduced AsA from its oxidized products (Asada, 1999; McKersie, and Leshem, 1994).

As an antioxidant, AsA can scavenge oxygen free radicals directly by reacting as a one-electron donor, due to the conjugate base (H^-) form of its ene-diol structure (Chaudière and Ferrari-Liou, 1999). Ascorbate can interact with $O_2^{\cdot-}$, $OH^{\cdot}$, and thiyl-radical forming MDHA when they are not scavenged by other systems (Asada, 1999).

In chloroplasts, ascorbate reduces H_2O_2 to water by a reaction catalyzed by APX, which completes the water-water cycle or photo-reduction of O_2 to water (Asada, 1999; Nakano and Asada, 1980). Two isoforms of APX have been identified in chloroplasts, thylakoid-bound (tAPX) and soluble stromal (sAPX) (Miyake and Asada, 1992; Miyake *et al*., 1993). Both are highly specific to their electron donor, ascorbate. In this regard, ascorbate regeneration in the water-water cycle is key to maintaining APX activity, as

this enzyme is the most sensitive to dysfunction, such as by excess light, in the water-water cycle (Asada, 1999; Nakano and Asada, 1980). It is also important to mention that this sensitivity to dysfunction is not the case for cytosolic APXs (cAPX), which scavenge H_2O_2 in other cell compartments (Asada, 1999).

Indirectly, ascorbate also participates in antioxidant recycling of tocopherol, converting the tocopheroxyl radical into α-tocopherol and monodehydroascorbate (MDHA) (McKersie and Leshem, 1994), and as a reductant in the thermal dissipation process exerted by the xanthophyll cycle, forming zeaxanthin (Yamamoto, 1979).

Once AsA is oxidized to MDHA, MDHA can dismutate back to ascorbate by photoreduced ferredoxin (Fd), which is associated to PSI complexes in the thylakoid membranes. Reduced Fd favors the reduction of MDHA instead of $NADP^+$ (Miyake and Asada, 1994). MDHA radicals can also be reduced back to ascorbate by MDHAR using NAD(P)H, a reaction most likely to occur in the stromal scavenging system where no reduced Fd is present (Asada, 1999).

There is another mechanism for reducing MDHA produced in the lumen by donation of electrons from ascorbate to PSII and PSI (Mano *et al.*, 1997) that involves a spontaneous reaction where MDHA is disproportionate it to ascorbate and DHA, which then diffuses to the stroma. The reaction rate of this disproportionation is highest at pH 3-6 (Asada, 1999). Once in the stroma, DHA is then rapidly reduced to AsA by DHAR using reducing equivalents (NADPH) from GSH before breaking down to tartrate and oxalate at pH 6 (Asada, 1999; Mano *et al.*, 1997; McKersie, and Leshem, 1994).

The accumulation of MDHA in leaves has been positively correlated with environmental stresses, such as UV-B (Hideg *et al*., 1997) and high light and chemicals (Heber *et al*., 1996). There is no information about this mechanism for fruit under stress.

Reduction-oxidation reactions involved in the recycling of AsA and GSH in the chloroplast are referred to as the Ascorbate-Glutathione cycle or Halliwell-Asada pathway (Fig. 1). This cycle also functions in the detoxification of H_2O_2 and oxidation of NADPH. McKersie and Leshem (1994) indicated that oxidation of NADPH, an energy-consuming process, could be viewed as "waste" of NADPH. However, in high light PSI transfers electrons to oxygen, which causes a high redox potential (high NADPH/NADP). Thus, the use of NADPH minimizes the reduction of oxygen by PSI.

According to Robinson and Bunce (2000), leaves of soybean and spinach plants submitted to drought stress maintained their ascorbate redox ratios (reduced AsA / total AsA+DHA) at 0.93-0.99. Nevertheless, severe water stress affected AsA and DHA levels, decreasing them by as much as 38% in greenhouse grown spinach. This is the most common response of ascorbate levels to oxidative stress (Smirnoff and Pallanca, 1996). However, in drought stressed soybeans grown in a growth chamber, ascorbate levels increased up to 20% (Robinson and Bunce, 2000), which could mean that the drought stress was not severe enough to cause damage, and therefore, the plant was able to acclimate. Smirnoff and Pallanca (1996) hypothesized that this stress response may have occurred because enzymes associated with the ascorbate-glutathione cycle (e.g. APX, MDHAR, GR) were able to efficiently maintain ascorbate in its reduced form by increasing enzymatic activity in response to a slow increase in water stress. This hypothesis has been corroborated in previous studies as an adaptive response to

excess ROS production, especially of H_2O_2, in water stressed plants (Smirnoff and Colombé, 1988; Smirnoff, 1993). Many studies have also shown that acclimation to high light involves an increase in the ascorbate pool (Gatzek *et al.*, 2002; Grace and Logan, 1996; Logan *et al.*, 1998b; Smirnoff, 2000).

An important approach for future enhancement of plant tolerance to oxidative stress could be the identification and overexpression of enzymes involved in ascorbate biosynthesis. Recently, Agius *et al.* (2003) reported a two-to three-fold increase in AsA content in transformed *Arabidopsis thaliana* overexpressing *GalUR*, a gene encoding D-galacturonate reductase. This enzyme catalyzes a key step in the biosynthetic pathway of L-ascorbic acid from D-galacturonic acid.

Reduced glutathione (γ-glutamylcysteinylglycine, GSH) is present in cells, subcellular compartments, and many tissues in higher plants. It is a tripeptide, Glu-Cys-Gly, where the sulfhydryl (SH) group of the cysteine (Cys) facilitates its antioxidant function (McKersie, and Leshem, 1994), mostly by transferring a hydrogen atom from the SH group, but also as a single-electron donor (Chaudière and Ferrari-Liou, 1999). The SH group, upon oxidation, reacts with another oxidized glutathione forming a disulphide bond and the compound glutathione disulfide (GSSG), which consists of two GSH molecules. The reduction of GSSG to GSH is catalyzed by the enzyme glutathione reductase (GR) (McKersie, and Leshem, 1994). This enzyme has an important role in photoprotection (reducing DHA in chloroplasts) as demonstrated by genetic engineering of plants (Aono *et al.*, 1995; Foyer *et al.*, 1991), and is prone to degradation under photooxidative conditions (Lascano *et al.*, 1998).

GSH concentrations vary with tissue age and growth environment, with higher levels forming in the light than in the dark. GSH can act as a free-radical scavenger, reacting with $^1O_2$, $O_2^{\cdot -}$, and $OH^{\cdot}$ radicals. In addition, GSH acts to stabilize membranes by removing peroxides (Price *et al.*, 1990) and is the reducing agent that recycles oxidized AsA through the enzyme DHAR. At pH 7 and GSH concentrations greater than 1 mM, GSH can also reduce DHA by a non-enzymatic mechanism (Foyer and Halliwell, 1976).

Other functions of GSH include the transport of reduced sulphur from leaves to sink tissues, detoxification of xenobiotics through its role as a substrate for the enzyme glutathione-S-transferase, conjugation to herbicides providing plant tolerance (e.g. of maize to triazine herbicides) (Timmerman, 1989), and as a precursor of phytochelatines (Ruegsegger *et al.*, 1990).

2. Enzymes

Among the antioxidant enzymes SOD has an important role in many cell organelles. It is an important metallo-enzyme forming part of the defense mechanism against oxidative stress (Bowler *et al.*, 1992; Salin, 1988a). It catalyzes the one-electron dismutation of the $O_2^{\cdot -}$ anion to H_2O_2 and oxygen by alternating reduction and oxidation of the metal coupled with the SOD protein (Salin, 1988b). Superoxide anions in chloroplasts come from the photo-reduction of O_2 occurring by the electron donation from the PSI complex, called the “stromal factor-mediated reaction”, which involves photo-reduced Fd or flavodehydrogenase MDHA reductase (Miyake and Asada, 1994).

The H_2O_2 generated by SOD is only a problem in the presence of a metal reductant [e.g. Fe(III) or Cu(II)] because, through reactions with Fenton reagents (also called Haber-Weiss reactions), H_2O_2 forms $OH^{\cdot}$ or alkoxyl radicals (Chaudière and Ferrari-Liou, 1999; Salin, 1988a), which are strong oxidizing (and toxic) compounds in biological systems (Czapski, 1984). In the case of $OH^{\cdot}$, there is no specific scavenging enzyme (Asada, 1999). While non-enzymatic dismutation of the $O_2^{\cdot -}$ anion is significant at physiological pH, the rate is much higher in the presence of SOD (Chaudière and Ferrari-Liou, 1999).

Three isoforms of SOD have been identified by their metal cofactors: copper/zinc (Cu/Zn), manganese (Mn) and iron (Fe). Cu/Zn-SOD is the major isoform of SOD found in chloroplasts of higher plants, with approximately 70% of it attached to the stromal side of the thylakoid membrane, where $O_2^{\cdot -}$ anions are being produced (Ogawa *et al.*, 1995). There are also, however, several cytosolic CuZn-SOD isoforms (Asada, 1999). The Mn-SOD isoforms occur in mitochondria and peroxisomes of eukaryotic cells (Salin, 1988b). The Fe-SOD isoforms have been reported to be present only in the chloroplast stroma of certain species, such as tobacco (*Nicotiana tabacum*; Kurepa *et al.*, 1997) and water lily (*Nymphaea luteum*; Salin, 1988b). Bowler *et al.* (1992) reported that each isozyme is independently regulated and that SOD activity increases under different environmental and xenobiotic stresses.

Many studies showed that overexpression of different SOD isoforms increased oxidative stress tolerance (Arisi *et al.*, 1998; Foyer *et al.*, 1994; Van Camp *et al.*, 1997), while others showed none or only a slight increase in tolerance (Payton *et al.*, 1997; Tepperman and Dunsmuir, 1990). These results suggest the concept of a complex,

interactive antioxidant system. For example, Foyer *et al*. (1994) reported that tobacco (*Nicotiana tabacum* var. Xanthi) plants transformed with the *E. coli* Mn-SOD gene showed a 30% increase in SOD activity, and a significant increase in tolerance to oxidative stress when plants were exposed to methyl viologen (active compound in the herbicide paraquat). Although Ogawa *et al*. (1997) reported that localization of chloroplastic CuZn-SOD played a key role in $O^{\cdot-}$ scavenging, even when they overexpressed Mn-SOD and targeted it to chloroplasts of tobacco plants deficient in chloroplastic CuZn-SOD, photobleaching and photoinhibition occurred under high light. This lack of protection may be because it was the improper SOD isoform in chloroplasts. In contrast, Kwon *et al*. (2002) reported a small increase in oxidative stress tolerance, with no visual photooxidative damage in transgenic tobacco plants overexpressing only genes of the CuZn or Mn SOD isoforms, but a significant increase in tolerance when these same SOD isoforms were overexpressed along with APX genes.

Catalase is a porphyrin-containing enzyme of high molecular weight, which is found in all aerobic eukaryotes. It catalyzes the dismutation of H_2O_2 in high concentrations into water and oxygen (Schonbaum and Chance, 1976). In contrast to APX, which reduces H_2O_2 in the Mehler-peroxidase and other reactions in the chloroplast, catalase has a high reaction rate (or V_{max}), but a low affinity for its substrate (H_2O_2), and does not utilize reducing power (Salin, 1988b; Willekens *et al*., 1997). Catalase is located mainly in peroxisomes, where it removes photorespiratory H_2O_2, but it is rarely located in chloroplasts. It is very sensitive to light and has a rapid turnover

rate. It was reported that under stress conditions (e.g. heat shock, salinity, and cold), catalase activity decreased (Hertwig *et al*., 1992).

Studies of Willekens *et al*. (1997), using transgenic tobacco plants possessing only 10% of the wild-type's catalase activity, demonstrated that this lack of catalase activity enhanced the plant's sensitivity to light stress (also to paraquat, salt, and ozone), which was visually seen as severe leaf necrosis. In addition, oxidized glutathione (GSSG) accumulated and a four-fold decrease of ascorbate occurred during this light stress. The authors, therefore, concluded that catalase plays a crucial role in sustaining cellular redox equilibrium during oxidative stress. They also indicated that catalase could be responsible for eliminating the majority of the H_2O_2 in leaf cells, with the remaining H_2O_2 eliminated by peroxidases. In this study, the authors identified the main source of H_2O_2 as being derived from photorespiration.

Logan *et al*. (1998b) also reported an increase in the activity of the antioxidant enzymes APX, SOD, GR, and CAT, as well as ascorbate content, in leaves of *Cucurbita pepo* L. and *Vinca major* L., when portions of the plants were suddenly exposed to high sunlight. All these changes prevented photooxidative damage in the tissue (measured as lipid oxidation products) and maintained PSII efficiency (pre-dawn Fv/Fm). Logan *et al*. (1998a) also found higher activities of APX and SOD, and higher concentrations of ascorbate, in plants acclimated to increasing sunlight.

3. Carotenoids

Carotenoids are an important group of isoprenoid pigments responsible for yellow, orange, and red pigmentation in many fruits (e.g. pineapple, orange, lemon,

strawberry, and tomato), leaves, flowers, birds, insects, and marine animals (Shi and Le Maguer, 2000). Chemically, there are two groups of carotenoids. The first group of carotenoids is comprised of highly unsaturated hydrocarbons that do not contain oxygen, such as lycopene, α-carotene, β-carotene, γ-carotene, and ξ-carotene. These are usually orange- and red-colored pigments. The second group of carotenoids, called oxycarotenoids (i.e. oxygenated derivatives) or xanthophylls, contain oxygenated groups on their terminal rings (Chaudière and Ferrari-Liou, 1999; Shi and Le Maguer, 2000).

Carotenoids play an essential role in oxygenic photosynthesis, and therefore, the survival of higher plants (Foyer, 1984; Yamamoto and Bassi, 1996). The carotenoids are mainly, but not exclusively, located in the PSI and PSII pigment-protein complexes of the thylakoid membranes. In each of these complexes there is a specific mixture of carotenoids, with lutein being a common component (reviewed by Yamamoto and Bassi, 1996). Carotenoids participate in: 1) light harvesting functions, which are especially important in low-light conditions; 2) protection of the reaction centers against photoinhibition under excess light; 3) regulation of PS II efficiency; and 4) protection of the entire photosynthetic system from photooxidative damage. In order to accomplish these structural and photophysical functions, specific carotenoids are located heterogeneously within photosynthetic pigment-protein complexes (Yamamoto and Bassi, 1996).

Carotenoids, as well as the sterol α-tocopherol, are hydrophobic ROS scavengers found in lipoproteins and membranes, although carotenoids can be present in aqueous solutions when associated with proteins or when they contain polar

functional groups (Britton, 1995). Carotenoids eliminate peroxyl radicals ($ROO^{\cdot}$), interrupting the propagation phase of lipid peroxidation, or they obstruct the formation of hydroperoxides from $^1O_2$ (Chaudière and Ferrari-Liou, 1999).

In vitro studies have shown that carotenoids may exhibit different scavenging mechanisms. One mechanism involves oxidation of the carotenoid molecule, as one electron from a carotenoid molecule is transferred to oxidizing radicals, thereby producing a cation radical, which can slowly dismutate (decay) non-enzymatically or be scavenged by α-tocopherol (Mortensen *et al.*, 1998). Another mechanism involves the direct addition of a free radical to the polyenic chain (Burton and Ingold, 1984; Britton, 1995). Carotenoids can also physically scavenge $^1O_2$ (Britton, 1995; Chaudière and Ferrari-Liou, 1999).

β-Carotene has important antioxidant functions in chloroplasts by protecting PSI and PSII, detoxifying ROS, and quenching $^3Chl^*$. β-Carotene and other carotenoids with $N \geq 9$ conjugated double bonds accomplish these functions by: 1) quenching excited chlorophyll molecules to prevent $^1O_2$ formation (main role), 2) reacting with lipid peroxidation products to terminate chain reactions (Burton and Ingold, 1984), and 3) scavenging $^1O_2$ and dissipating the energy as heat, although this event is believed to be limited by diffusion (Foyer, 1984; Mathis and Kleo, 1973; Yamamoto and Bassi, 1996). The mechanism by which carotenoids quench $^3Chl^*$ and $^1O_2$ is not completely understood, because the energy level of the carotenoid triplet state ($N=9$) is insufficient to quench $^3Chl^*$ and $^1O_2$ (Yamamoto and Bassi, 1996). It is estimated that the excess energy transferred from chlorophyll molecules to carotenoids under light saturated conditions accounts for only 20% of the energy dissipated in chloroplasts (Foyer, 1984).

In some algae, such as *Dunaliella bardawil*, accumulation of inter-thylakoidal β-carotene, which is not near the reaction centers, occurs as a response to environmental stresses, such as high light, extreme temperatures, and UV-A, but not UV-B, radiation (White and Jahnke, 2002). These authors found a positive correlation between β-carotene level and photoprotection against damaging UV-A radiation, and so they concluded that β-carotene acts to filter this type of light. UV-A treatment also increased APX activity and ascorbate concentration in this algal species.

Zeaxanthin is another carotenoid, which is implicated in thermal energy dissipation by its participation in the xanthophyll cycle under both excess and normal sunlight conditions. Its mode of action has been reviewed by Demmig-Adams and Adams III (1992a, 1996a). Briefly, it facilitates dissipation of excess energy, as heat, from light-harvesting chlorophyll molecules, thus preventing the formation of ^{3}Chl* at the reaction centers. This process occurs in the light-collecting antenna complexes, particularly in the minor proximal antenna that connects the major light-harvesting complex II (LHCII) and the PSII core (reviewed by Yamamoto and Bassi, 1996). The thermal energy dissipation process is measured as non-photochemical quenching (NPQ, or sometimes as q_N or q_{NP}) of chlorophyll fluorescence, which results from three principal mechanisms: 1) pH-dependent energy dissipation caused by light-induced intra-thylakoid acidification or “energy-dependent” quenching, q_E; 2) “state 1 - state 2” transition quenching. q_T, due to phosphorylation of LHCII; and 3) photoinhibition of photosynthesis or “photoinhibitory” quenching, q_i (Krause and Weis, 1991). The first mechanism is the major contributor to NPQ, and it can be easily identify kinetically because it is rapidly induced and reversible in darkness due to the disappearance of

light-driven proton translocation across thylakoid membranes (Niyogi *et al*., 1998). In recent years, this mechanism has been verify through an *Arabidopsis* mutant (i.e. *npq4-1*, *npq4-8,* and *npq4-9*) containing a knockout of the PsbS gene showing loss of q_E; it encodes the PSII-S protein (CP22), a pigment-binding protein associated with PSII in higher plants (Li *et al*., 2000). This protein has a specific role in the NPQ mechanism (Li *et al*., 2000; Peterson and Havir, 2001). Li *et al*. (2002) reported that the overexpression of PsbS gene increased feedback de–excitation (qE). They concluded that the species-specific expression of the PsbS gene could be an essential regulatory component or switch for qE. Therefore, together with de-epoxidized xanthophylls and low thylakoid lumen pH, PsbS is a key component of NPQ or thermal dissipation. They suggested also that genetic manipulation of this enzyme could increase plant tolerance to environmental stresses by enhancing resistance to photoinhibition.

This non-radiative energy dissipation mechanism operates in PSII. It involves the conversion of violaxanthin (V) to antheranthanthin (A; an intermediate form), and finally to zeaxanthin (Z) under excess light conditions, and the reverse reactions (Z into V) under low light conditions. Two enzymes catalyze these reactions: 1) violaxanthin de-epoxidase (VDE), which catalyzes de-epoxidation of V to Z, through A, is activated by low pH (below 6.5) in the lumen of the thylakoid (Pfündel and Dilley, 1993; Hager and Holocher, 1994), and requires AsA as reductant (Hager and Holocher, 1994); and 2) zeaxanthin-epoxidase (ZE), which catalyzes the reverse reaction, is located at the stromal side (Hager and Holocher, 1994), and requires oxygen, NAPDH, and a higher optimum pH (Demmig-Adams and Adams III, 1992a). These reactions can be detected

in vivo by a light-induced absorbance change at 505 nm in green leaves, which reflects the rate of violaxanthin de-epoxidation (Bilger *et al.*, 1989).

Niyogi *et al.* (1998), utilizing *Arabidopsis* xanthophyll cycle mutants (i.e. *npq1*, VDE gene mutated, and *npq2*, ZE gene defective), demonstrated that de-epoxidation of V to Z is required for most NPQ in *Arabidopsis*. The loss of this capacity for NPQ in *npq1* increased its sensitivity to photoinhibition. Zeaxanthin accumulation in *npq2*, however, did not affect overall NPQ, but did delay the induction and relaxation of the process. This resulted in slower reversibility of NPQ, which could decrease photosynthetic efficiency under normal or low light levels.

Lower ascorbate availability also can reduce VDE activity, and therefore, NPQ. According to *in vitro* studies by Neubauer and Yamamoto (1994), the Mehler-peroxidase reaction competed with VDE for ascorbate, which finally reduced q_E. Therefore, ascorbate may be a limiting factor for NPQ *in vivo*. This hypothesis was tested and proven by Müller-Moule *et al.* (2002), who investigated NPQ induction in an ascorbate-deficient mutant of *Arabidopsis* (i.e. *vtc 2-2*). These authors concluded that ascorbate deficiency in this mutant must be mostly localized in chloroplasts; otherwise, plant functions other than NPQ would have been affected. The mutant showed less NPQ than the wild type; however, quantum yield of PSII was the same. Therefore, the authors also concluded that the ascorbate limitation was responsible for the NPQ phenotype of the mutant. This conclusion was also supported by ascorbate feeding experiments to individual leaves of the mutant, which raised NPQ levels of the mutant to near that of the wild type. The results of Müller-Moule *et al.* (2002) indicated that the ascorbate limitation of *vtc2-2* also directly affected VDE activity (found specifically in chloroplasts),

and therefore, the xanthophyll cycle, which already competes with APX for ascorbate. Similar competition for ascorbate was also found under normal light conditions. Therefore, even in wild-type plants, especially when they were subjected to oxidative stress, there was an increased risk that reduced ascorbate wouldl become limiting for VDE activity and NPQ.

The pool size of xanthophylls varies with other components of the photosynthetic apparatus as the light environment changes, in a phenomenon known as acclimation. In response to the perceived need for greater photoprotection and dissipation of excess light energy, plant leaves in high-light environments increase their xanthophyll pool size relative to leaves in low-light environments (Demmig-Adams and Adams III, 1992b; Demmig-Adams and Adams III, 1996b; Logan *et al*., 1998a). This same acclimation response also has been reported when sudden changes from low to high light conditions occur (Logan *et al*., 1998b). Under high-light conditions, xanthophylls may constitute more than 30% of total carotenoids (Demmig-Adams and Adams III, 1992b). In this study, β-carotene followed the same pattern of accumulation as xanthophylls, but α-carotene accumulated mainly in the light. In contrast, neoxanthin and lutein accumulation did not respond consistently to light intensity (Demmig-Adams and Adams III, 1992b).

Besides reducing photochemical efficiency, environmental stresses can cause adjustments to the xanthophyll pools. Demmig *et al*. (1988) reported an increase in Z levels and xanthophyll cycling in plants submitted to water stress, as well as a reduction of chlorophyll fluorescence (i.e. increased photoinhibition) due to elevated thermal energy dissipation and photoprotection. Nitrogen deficiency also has resulted in

increased Z content (Khamis *et al*., 1990), while iron deficiency has increased the molar ratios of xanthophylls to chlorophyll molecules (Morales *et al*., 1990). Morales *et al*. (1990) also reported that plants submitted to iron deficiency showed a decrease in neoxanthin, β-carotene, and chlorophyll a contents. Plants subjected to low temperature stress, that is when grown for long periods in the cold, accumulate large amounts of Z, because epoxidation of Z to V is inhibited at low temperatures. This indicates that the thermal energy dissipation system mediated by the xanthophylls is continuously engaged under these conditions (Demmig-Adams and Adams III, 1992a).

A genetic approach to enhancing carotenoids to increase oxidative stress tolerance in plants has received attention lately. Davison *et al*. (2002) reported that the overexpression of only one enzyme, β-carotene hydroxylase (*chy*B), increased stress tolerance. This thylakoid-bound enzyme participates in the synthesis of xanthophyll from β-carotene. Its overexpression increased the V pool in the dark from 14-22% to 30-40% of total carotenoids, without affecting biosynthesis of other carotenoids, except for the depletion of β-carotene. The extra V was localized in the thylakoid membranes, and under excess light it was converted to Z at double the rate of the wild-type and with no change in average de-epoxidation state. These authors, however, did not observe an increase in the capacity of NPQ in *chy*B plants, as would have been expected, so they concluded that the higher stress tolerance of *chy*B was provided by the increased capacity of Z to scavenge ROS, thus preventing lipid peroxidation.

Lycopene ($C_{40}H_{56}$) is one of the most efficient singlet-oxygen quenchers among the carotenoids, showing high antioxidant activity and the highest quenching rate constant (Kq) of all carotenoids (Shi and Le Maguer, 2000). This characteristic and its

red color are a consequence of its acyclic structure and the linear array of its conjugated double bonds. Lycopene, however, shows very high hydrophobicity (Shi and Le Maguer, 2000).

In tomato fruit, lycopene starts to accumulate as microcrystalline aggregates in chromoplasts after chloroplast-chromoplast transformation takes place. This transformation takes two or three days to complete, after which the plastids begin to lose starch and chlorophyll and accumulate phytoferritin, plastoglobules, and crystalline lycopene (Simpson *et al*., 1976). Lycopene quenches $O_2^{\cdot -}$ and traps peroxyl radicals ($ROO^{\cdot}$) (Shi and Le Maguer, 2000).

Lycopene oxidation can occur at either end of the C-40 skeleton by scission of any double bond, thus degrading it so that it is no longer a carotenoid. The possible products of lycopene degradation are norbixin, apo-6-lycopenal, and 2-methyl-2-hepten-6-one, the last two formed during lycopene photosensitization (Shi and Le Maguer, 2000).

4. Other compounds

Another antioxidant exerting a protective role against photooxidative damage in chloroplast membranes is α-tocopherol (αTOH), or vitamin E, which is also an efficient scavenger of $^1O_2$ and lipid peroxyl radicals ($ROO^{\cdot}$) (Foyer, 1984; Fryer, 1992). The antioxidant function of αTOH is particularly important in thylakoid membranes. Here, αTOH is located near the surface of the membrane bilayer, where it can readily diffuse laterally to scavenge $ROO^{\cdot}$ through hydrogen atom transfer. Once oxidized by this antioxidant activity, the chromanoxyl radical ($\alpha TO^{\cdot}$) is reduced to αTOH in the aqueous

phase by AsA (McKersie, and Leshem, 1994). The hydroperoxide formed (LOOH) must be reduced by glutathione peroxidases to complete the antioxidant process (Chaudière and Ferrari-Liou, 1999). α-Tocopherol has also been found in non-photosynthetic tissues of higher plants (McKersie, and Leshem, 1994).

Although phenolics have been historically related only to plant defense against herbivores, Close and McArthur (2002) recently presented evidence for the involvement of phenolics in photoprotection of leaves. The antioxidant capacity of phenolics has been shown for flavonoids (Grace *et al.*, 1998; Rice-Evans *et al.*, 1996) and anthocyanins (Wang *et al.*, 1997), which can act against AsA oxidation (Rice-Evans, 1996; Sarma *et al.* 1997). Plant phenolics, especially flavonoids, can inhibit lipid peroxidation (Halliwell, 1995). Phenolic accumulation in leaves, as well as ascorbate and glutathione, respond to environmental conditions, such as light intensity and nutrient availability (Bryant *et al.*, 1987; Grace *et al.*, 1998; Mole *et al.*, 1988), and different stress conditions that lead to increased risk of oxidative damage (Close and McArthur, 2002).

Phenolics absorb strongly in the UV-B region of the electromagnetic spectrum, but also in the visible region, and therefore, partially reduce the deleterious effects of these wavelengths by acting as sunscreens (Jordan, 1996; Veit *et al.*, 1996). Moreover, there is evidence indicating that radiation filtering performed by anthocyanins affects photosynthetic rates of leaves by maintaining photosynthetic efficiency at high light levels and low temperatures (Pietrini *et al.*, 2002).

Changes in flavonoid synthesis under varying UV-B conditions have been reported to occur within hours (Veit *et al.*, 1996). In this study, flavonoid contents in

plants exposed to natural UV-B radiation changed diurnally, increasing from dawn to midday and then decreasing throughout the rest of the day.

The *orth*o-dihydroxylated flavonols, such as quercetin glycosides (Q), which have been shown to increase preferentially under enhanced UV-B radiation (Ryan *et al*., 2002), are more effective antioxidants than their mono-hydroxylated counterparts, such as kaempferol glycosides (K) (Montesinos *et al*., 1995). Furthermore, Ryan *et al*. (2002) reported that the increased Q:K ratio correlated with photoprotection events in plants subjected to enhanced UV-B radiation. The up-regulation of the enzyme flavonoid-3'-hydroxylase, which catalyzes the conversion of mono-hydroxylated flavonoids to their correspondent *ortho*-dihydroxylated flavonoids, was purportedly responsible for this photoprotection.

C. Fruit Antioxidant Systems

Most of the research on antioxidant systems in plants has been done in leaves, and only limited research is available for fruit. However, all of the antioxidant systems that are found in leaves are also reported to be present in fruit of the species studied. There has been contrasting evidence of changes in antioxidant levels during fruit development and maturation within and among species, although increased oxidative stress with fruit maturity and ripening has been reported (Lacan and Baccou, 1998; Rogiers *et al*. 1998; Wang and Jiao, 2001). For example, in pears (*Pyrus communis* L. cv. Conference), AsA and GSH concentrations decreased with increasing fruit maturity (Lentheric *et al*., 1999). In the same study, APX activity increased 2.5-fold in the more mature fruit, while GR remained stable and CAT decreased. In contrast, Jimenez *et al*.

(2002) reported that reduced and total glutathione and ascorbate concentrations increased during ripening in tomato (*L. esculentum* Mill. cv. Alisa Craig). They also concluded that changes in CAT, SOD, and recycling enzyme activities (APX, MDHAR, DHAR, and GR) during fruit maturity indicated that these antioxidant systems play an important role in the ripening processes of tomato fruit. On the other hand, Andrews *et al*. (2004) and Torres (2001), working with different genotypes of tomato, found that AsA increased from the immature green stage of fruit until an intermediate maturity stage when fruit were turning from green to yellow, after which AsA declined as the fruit ripened. As in the study of Jimenez *et al*. (2002), Torres (2001) found that total glutathione (GSH+GSSG) concentrations in the skin increased continuously from immature green to ripe fruit. Andrews *et al*. (2004), however, found stable glutathione concentrations throughout fruit development. In this study they also found large increases in SOD, APX, and MDHAR activities during fruit development.

In sweet pepper (*Capsicum annum* L. cv. Golden Bell), Imahori *et al*. (1998) reported also that ascorbate concentrations increased during fruit maturation. They also found that ripe yellow fruit had the lowest APX activity. DHAR activity remained constant during maturation, and MDHAR increased from the green to the yellow fruit stage.

In addition to providing support for the hypothesis that fruit maturation involves increasing oxidative stress resulting from lipid peroxidation, in a study of saskatoon (*Amelanchier alnifolia* Nutt.) fruit Rogiers *et al*. (1998) found decreased SOD and CAT activities during maturation. They concluded that the reductions in the activities of these enzymes occurred due to the accumulation of ROS, specifically H_2O_2, during ripening. They also observed higher GSH and GSSG concentrations, GR activity, and ratio of

GSSG / total glutathione, all of which are indicators of increasing oxidative reactions during fruit ripening.

On the other hand, the research of Wang and Jiao (2001) suggested that as blackberry (*Rubus* sp.) fruit matured and ripened, its capacity to remove ROS decreased. This was due to reductions in the ascorbate and glutathione pools, and decreasing activities of antioxidant enzymes, such as SOD, CAT, APX, MDHAR, and DHAR. They also concluded that maturation occurred along with an oxidative burst, which they measured as a reduction in unsaturated fatty acids, implying lipid peroxidation.

Du and Bramlage (1994) found great variability in SOD activity among apple cultivars and throughout fruit senescence. They also reported that SOD activity increased in fruit affected by the physiological disorder “senescent scald”. Gong *et al.* (2001) found a decrease in SOD activity as ‘Braeburn’ apples ripen.

IV. Effects of Photooxidative Stress on Fruit Antioxidant Systems

Changes in antioxidant components in fruit tissue have only been examined partially and not thoroughly enough to investigate their function on physiological disorders that are caused by oxidative stress, such as sunscald. Some of these studies are briefly described.

Under natural conditions, Andrews and Johnson (personal communication) found higher levels of AsA in fruit peel of moderately sunscald-damaged ‘Fuji’ apples than in uninjured fruit peel. They also found more AsA in peel from the exposed side of the apple than peel from the shaded side. They reported that seasonal changes in AsA and

GSH concentrations coincided with differences in sunscald susceptibility. These authors and others also detected higher SOD activity in apple skin exposed to direct solar radiation than shaded skin of the same fruit (Andrews and Johnson, 1996; Yuri *et al.*, 1998). In contrast, Prohens *et al.* (2004) found a decrease in ascorbate levels as sunscald incidence increased over time in detached pepino fruit. The same response was reported by Adegoroye and Jolliffe (1987) for tomato fruit. None of these reports were accompanied with antioxidant enzymes activities, especially those involved in ascorbate recycling.

Using a different approach, a study conducted by Adegoroye and Jolliffe (1987) demonstrated that tissue from detached tomato fruit exposed to radiation from incandescent lamps (650 W/m^2) and 40°C air temperature for 1.5 hours (causing incipient fruit injury) and 4 hours (causing advanced injury) had lower AsA levels than that of the control. The authors also found that treated tomatoes showed blockage of lycopene accumulation and protopectin solubilization.

An increases in total phenolics, flavonoids, and changes in pigment composition have also been reported to occur in fruit exposed to high solar radiation and/or affected by photooxidation or sunscald (Merzlyak *et al.*, 2002). Pigment profiles were shown to depend on cultivar in the case of apples. Solovchenko and Schmitz-Eiberger (2003) and Merzlyak and Solovchenko (2002) found lower amounts of chlorophyll and carotenoids in the peel on the exposed side of ‘Granny Smith’ fruit, which does not accumulate anthocyanins, whereas, ‘Braeburn’ fruit peel, which does accumulate anthocyanins, showed higher amounts of these pigments. The authors attributed these cultivar differences to different adaptation mechanisms to high light, with decreased chlorophyll

in ‘Granny Smith’ to reduce photooxidative stress and anthocyanin accumulation in ‘Braeburn’ to protect the photosynthetic apparatus. These investigations did not consider that ‘Granny Smith’ is a cultivar that is prone to photobleaching, which translates into lower chlorophyll concentrations in exposed fruit.

Solovchenko and Schmitz-Eiberger (2003) also reported that the accumulation of quercetin glycosides in exposed skin of ‘Braeburn’ apples was responsible for the stability of the Fv/Fm ratio at 0.7, and therefore, optimum functioning of the photosynthetic apparatus in the course of increasing UV-B irradiation. They did not observed the same pattern of quercetin glycoside accumulation in ‘Granny Smith’ apples, whose quercetin glycoside concentration was half that found in ‘Braeburn’. They concluded that there was a positive correlation between quercetin glycoside accumulation and UV-B dose, and suggested that this was an acclimation response of ‘Braeburn’ apples to high solar radiation environments. Similar results were reported by Merzlyak *et al.* (2002) in different red-colored apple cultivars.

V. Current Knowledge of Photooxidative Stress and Injury in Fruit

The schematic (Fig. 2) below summarizes all of the currently known factors involved in photooxidative stress in fleshy fruit. The processes are believed to be similar to those involved in photooxidative stress of leaves, but there is still very little supporting scientific evidence. There are data on temperature profiles of fruit exposed to direct solar radiation, and controlled environment studies where it was shown that both high light and temperature were required to induce sunscald damage. There have only been limited data on the effects of UV radiation on sunscald, and these have been generated

only under controlled laboratory conditions. The contribution of UV radiation to photooxidative stress in fruit, therefore, is still in question.

Visible symptoms of sunscald have been well described; however, except for electrolyte leakage from ruptured cells, the physiological responses that are associated with these visible symptoms have not been elucidated. Under the environmental conditions that induce photooxidative stress in fruit, a decrease in the intrinsic efficiency of PSII (Fv/Fm) is expected when chlorophyll-containing fruit are exposed to high light. This decrease in PSII efficiency should eventually lead to an increase in ROS in response to stressful environments. Neither of these responses has been documented for fruit in response to photooxidative stress. Eventually, damage to DNA, proteins, and lipids is expected to occur.

Since photooxidative stress is believed to eventually lead to sunscald, there has been some research on specific metabolites, including AsA and flavonoids, but a paucity of information is available on antioxidant enzymes, which are critical for maintaining antioxidant metabolites in their reduced state.

“Static” photo-protection is achieved when photooxidative stress is moderate, and involves flavonoids and anthocyanins. These secondary metabolites accumulate in cell vacuoles of exocarp tissue and act as solar filters, absorbing not only UV radiation, but also visible light. In this way, they provide a direct photo-protective mechanism to the photosynthetic apparatus under high light conditions.

“Dynamic” protection refers to the active functions performed by antioxidant metabolites (e.g. AsA and GSH) and enzymes (e.g. SOD, APX, and GR) during oxidative stress by scavenging ROS scavenging and/or recycling antioxidant

metabolites. Flavonoids may also provide "dynamic" protection against photooxidative damage.

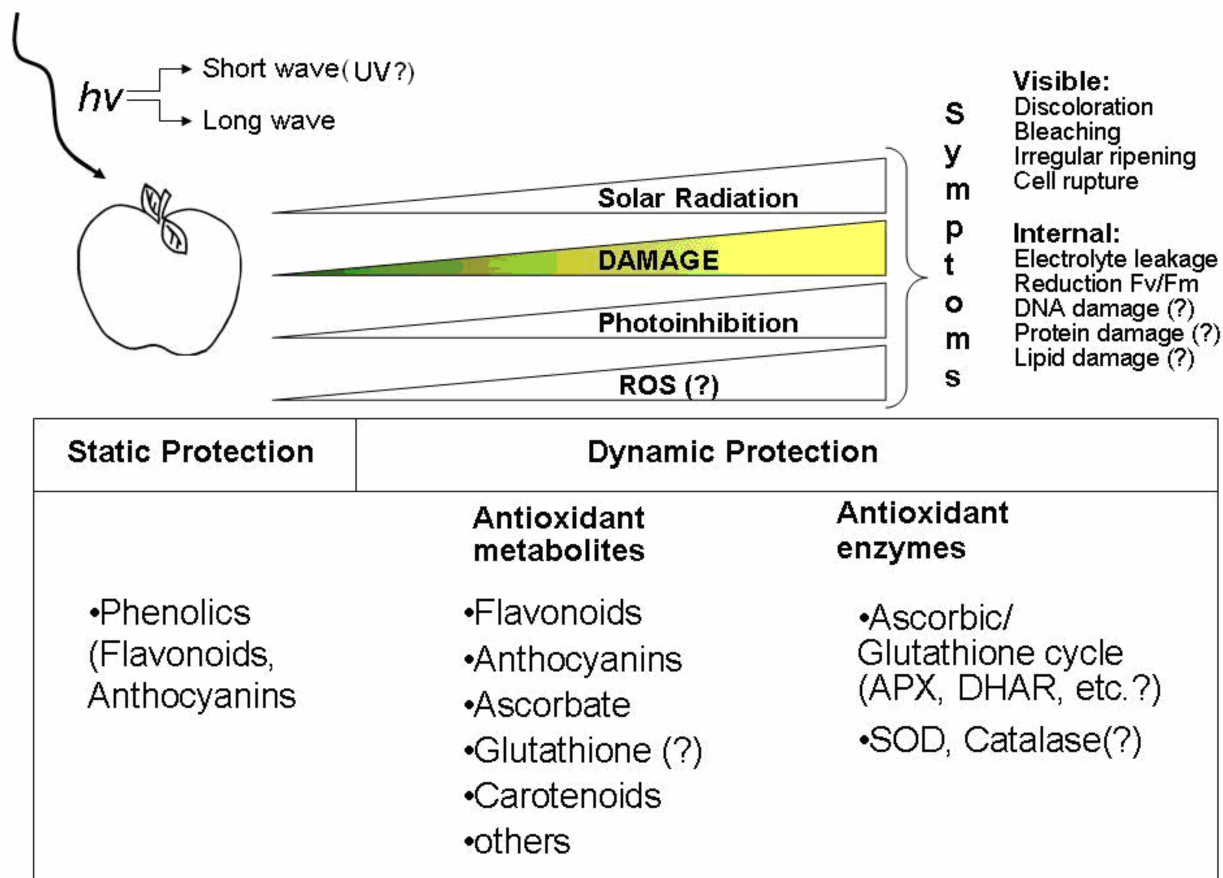

Figure 2. Environmental (solar radiation), physiological (photosynthetic efficiency) and biochemical factors (static and dynamic protection) involved in photooxidative damage or sunscald development in fleshy fruits. Question marks indicate non-studied or unclear results available in the literature.

VI. Conclusions and Future Research Directions

Photooxidative damage or sunscald of fruit is an important physiological disorder that causes millions of dollars of crop losses in arid and semiarid regions of the world. The factors responsible and mediators of the problem are due to oxidative stress as a result of the high solar radiation and temperature in the geographical locations where these fruit crops are grown. The current evidence is that both solar radiation and high temperatures are necessary for photooxidation to occur and sunscald to develop. There is inconclusive evidence concerning the involvement of UV radiation as a contributing factor in sunscald development. Since sunscald is a type of oxidative stress, some research has focused on specific antioxidant molecules, such as AsA and flavonoids, in the sun-exposed versus shaded side of fruit. Nevertheless, there has still not been a complete biochemical characterization of this physiological disorder, including a general view of all the antioxidant systems present in fruit cells.

In the future, molecular tools should be applied to study gene expression and identify important proteins involved in the progression of photooxidative stress events of sunscald. This approach might identify those components that could be used as molecular markers for breeding tolerant cultivars for the many fruit crop species grown in high light and high temperature environments. The information could also be useful for formulating effective ”solar protectants”, which could be applied during periods of high fruit susceptibility to photooxidative damage. An eventual indirect benefit of this research could be added nutritional value to these food crops for human health and disease.

In the meantime, several cultural methods can be used to control sunscald. Some of these practices either have been or are currently being used for many fruit crops, including: 1) evaporative cooling with sprinklers, 2) shade covers, 3) fruit bagging, and 4) chemicals applications of clay-based, particle-film reflectants. The use, cost, and effectiveness of sunscald reduction vary among these methods, but all of them are only economically viable for high value crops and cultivars.

As a general rule, low or reduced foliage density, either due to genetics, cultural practices (e.g. pruning and leaf removal), or pests and diseases, lead to an increase sunscald incidence. Clearly, the least expensive method of control is the choice of the proper genotype and a sound planting design that is matched to the topography and microclimate of the site. Ideally, achieving a proper balance of vegetative and fruit growth and development, which utilize leaves as the least expensive and most effective protection strategy, especially for sunscald-susceptible cultivars, is the best crop management.

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CHAPTER 2

PHYSIOLOGICAL AND METABOLIC RESPONSES OF FRUIT EXOCARP OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL) MUTANTS TO NATURAL PHOTOOXIDATIVE CONDITIONS

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ABSTRACT

Photooxidative damage to fruit occurs during high solar irradiance and results in sunscald with surface discoloration and bleaching. Using a system that permitted the imposition of photooxidative stress under natural solar radiation, we evaluated surface color changes, photosynthetic efficiency, and pigment and flavonoid composition in exocarp of immature green tomato (*Lycopersicon esculentum* Mill) fruit. Exposed or covered sections of detached fruit of mutants (*anthocyanin absent*, *β-carotene*, *Delta*, and *high pigment-1*) with attenuated pigment and/or antioxidant metabolite levels, and their nearly isogenic parents ('Ailsa Craig' and 'Rutgers'), were subjected to five hours of high solar irradiance, either in the presence or absence of ultraviolet (UV) radiation. Photooxidative stress on detached tomato fruit reproduced typical sunscald symptoms which were observed on attached fruit. Both high temperature and solar irradiance caused fruit surface discoloration with faster degradation of chlorophyll (Chl) than carotenoids (Car), leading to an increase in the Car/Chl ratio. Bleaching of the fruit surface was mostly caused by visible light, whereas elevated temperatures were mostly responsible for inactivation of photosynthesis, measured as a decreased in F_v/F_m. Among flavonoids, quercetin and kaempferol concentrations increased rapidly upon exposure to sunlight, but not to natural UV radiation, suggesting rapid photo-protection in response to visible light. Interestingly, naringenin synthesis was not induced upon exposure to sunlight. Fruit exocarp from *high pigment-1* had higher Chl and Car levels than the other genotypes, and increased quercetin and kaempferol concentrations compared to 'Ailsa Craig', partially explaining its apparent greater tolerance to photooxidative stress.

Key words: fruit, oxidative, photooxidation, photosynthesis, stress, sunscald

INTRODUCTION

In fruit, photooxidative damage refers to photodynamic injury of heated tissue that occurs under conditions of intense sunlight and elevated temperatures (Barber & Sharpe 1971). Commonly, this photodynamic injury is called sunscald, with symptoms occurring as various types of surface discolorations. Sunscald damage can be severe enough to cause economic losses in several fruit crops, including apple (Brooks & Fisher 1926, Moore & Rogers 1943; Andrews & Johnson 1996; Yuri, Vásquez, Vásquez & Torres 1996), banana (Wade, Kavanagh & Tan 1993), melon (Lipton 1977), peach (Moore & Rogers 1943), pepper and cucumber (Ramsey, Wiant & McColloch 1952; Barber & Sharpe 1971; Rabinowitch, Ben-David & Friedmann 1983; Fallik, Ziv, Grinberg, Alkalai & Klein 1997), tomato (Moore & Rogers 1943, Ramsey *et al*. 1952; Retig & Kedar 1967; Rabinowitch, Kedar & Budowski 1974), and raspberry (Renquist, Hughes & Rogoyski 1989).

In the fruit of cultivated tomato (*Lycopersicon esculentum* Mill), photooxidative damage occurs mainly in the green epidermal and hypodermal tissues, although “breaker” fruit (intermediate maturity stage) is the most susceptible (Rabinowitch *et al*., 1983). Photooxidative stress in fruit may be confounded with normal ripening, because oxidative processes have been implicated in the ripening process (Blackman & Parija 1928; Brennan & Frenkel 1977; Jiménez, Creissen, Kular, Firmin, Robinson, Verhoeyen & Mullineaux 2002; Andrews, Fahy & Foyer 2004).

Although both high irradiance and high temperatures are necessary to cause sunscald symptoms, there has been only loose evidence addressing the importance of each of these environmental factors individually, as well as the role of ultraviolet (UV) irradiance on photooxidative injury of fruit.

The present work is the first part of a thorough physiological and biochemical study of the effects of high solar irradiance on the development of photooxidative injury in tomato fruit. In this study, we implemented a system to test fruit susceptibility to photooxidative stress using natural solar radiation. The specific function of different antioxidant system components have been examined by using fruits of specific tomato mutants with attenuated pigment and/or antioxidant metabolite levels. Our hypothesis is that modulation of these compounds in the fruit will result in differential tolerance to photooxidative damage following exposure to natural high visible and/or UV irradiance at elevated temperature.

MATERIALS AND METHODS

Tomato mutants

The tomato (*Lycopersicon esculentum* Mill) genotypes used in this experiment are: 1) *anthocyanin absent* (*aa*) (LA 3617), with mutation in chromosome 2 and anthocyanin completely absent in all plant parts (C.M. Rick Tomato Genetics Resources Center; Univ. of California, Davis, CA, USA). 2) *β-carotene* (*B*) (LA 3179), with mutation at loci 6 and 106 (Stevens & Rick, 1986), high β-carotene, low lycopene in ripe fruit, bright orange mature fruit (Harris & Spurr 1969; Ronen, Carmel-Goren, Zamir & Hirschberg 2000; Tomes, Quackenbush & Kargl 1956, 1958). The *B* gene (B/B alleles)

is allelic in a locus encoding a fruit and flower-specific lycopene β-cyclase that shifts the carotenoid pathway from lycopene to β-carotene synthesis (Ronen *et al.*, 2000). 3) *Delta* (*Del*) (LA2996A), with inhibited lycopene, increased δ-carotene (Williams, Britton & Goodwin 1967), reddish-orange mature fruit. 4) *high pigment* (*hp-1*) (LA2838A, *hpA* and LA3004, *hpR*) is a recessive non-allelic mutation at locus 12 that was first identified in 1917. Chlorophyll, carotenoids (lycopene and β-carotene), and ascorbic acid (AsA) contents of fruit are intensified (Andrews *et al.*, 2004; Baker & Tomes 1964; Clayberg, Butler, Kerr, Rick & Robinson 1970; Jarret, Sayama & Tigchelaar 1984; Stevens & Rick, 1986; Thompson 1955, 1961; Torres, 2001), as well as anthocyanins (Kerckhoffs, De Groot, Van Tuinen, Schreuder, Nagatani, Koornneef & Kendrick 1997; Wettstein-Knowles, 1968a, 1968b). The mutation is located on a negative regulator(s) of phytochrome signal translation, which causes amplified photo-responsiveness and pleitropic effects (Jarret *et al.*, 1984; Kerr 1965; Kerckhoffs *et al.*, 1997; Peters, Széll & Kendrick 1998). All mutants are nearly isogenic in the cultivar 'Alisa Craig' (AC), except LA3004, which is nearly isogenic in 'Rutgers' (R).

Seeds from the previously described tomato mutants and their parents were obtained from the C.M. Rick Tomato Genetics Resource Center (University of California, Davis, CA).

Field site

The research site was located in Lewiston, Idaho (46°23' N; 116°59' W) at an elevation of 430 m above sea level. The site was level and had a uniform soil of Nez Perce silty, clay-loam texture (fine, montmorillonitic, mesic Xeric Argialbolls). This region

is classified as desert steppe with summer (June-Sept.) mean maximum temperatures of 30°C on generally cloudless days, as most precipitation occurs only in the winter months.

Seedlings of the tomato genotypes were started in cell packs in a greenhouse until they had several true leaves, at which time they were transplanted in a replicated, randomized complete block design in the field, consisting of 4 blocks with six plants per plot. Plants were spaced 1.2 m apart in the rows, with rows 2.4 m apart.

All plants were irrigated with buried drip tape, one hour per day during the first 30 days after planting, and two hours per day from 30 days after planting until the end of the growing season. The output of each emitter was 1.9 L hr $^{-1}$. Plants were surface fertilized two weeks after transplant with calcium nitrate (30 g per plant).

Experimental Setup

Determination of susceptibility to photooxidative damage was evaluated by exposing detached immature green fruit from all genotypes to treatments of natural sunlight, either with (+UV) or without UV exposure (–UV, by the use of 0.635 cm safety glass plate filter), for three durations of exposure (i.e. 0, 2.5, and 5 h) (Fig. 1A). In order to investigate the independent effects of solar radiation and high temperature on photooxidative injury, each fruit from the treatments previously described was partially covered with reflective tape allowing air movement on the fruit surface beneath (Fig. 1B).

Each treatment was represented by a group of four similar sized fruits, harvested from un-exposed locations in the plant canopy just before setting up the experiment,

with a total of 168 fruit tested per date. Harvested fruits were placed on top of a white board with the calyx-end up (Fig. 1A). The set of treatments described previously were replicated on five separate dates in 2003: 17, 22, and 29 July and 13 and 26 Aug. On all dates, experiments were conducted between 11:30 a.m. and 5:00 p.m. Pacific Daylight Savings time. Each date was considered a block.

Field measurements

Chlorophyll fluorescence was measured using a portable chlorophyll fluorometer (OS-500, Opti-Science, Tyngsboro, MA). The fluorescence parameters calculated to evaluate intrinsic photosystem II (PSII) efficiency during photooxidative stress was $(F_m-F_O)/F_m$ (F_v/F_m) where F_O and F_m are minimal and maximal fluorescence yield of a dark adapted measurement. Dark-adapted fruit measurements were taken after 30 min of darkening the tissue. For this purpose, an additional fruit was provided from each treatment and measured three times on each of the experimental dates.

Objective color change was measured using a colorimeter (Minolta CR-300, Ramsey, NJ, USA). Data were expressed in CIELAB units where L* indicates lightness, a* blue-green/red-purple and b* yellow-blue hue components. The a* and b* coordinates were used to determine ”hue angle” (b*/a*, 0°- 360°) or color, and ”chroma” [C, $(a^{*2} + b^{*2})^{1/2}$] or color saturation or intensity (McGuire, 1992). Measurements were taken in triplicates per fruit on all experimental dates.

Solar radiation (total; photosynthetic photon flux density, PPFD; and UV) was measured throughout the experiments. Total radiation (400-1100 nm) was measured using a pyranometer (LI-200SA, LI-COR, Lincoln, NE, USA), PPFD (400-700nm) was

measured using quantum sensors (LI-190SA, LI-COR, Lincoln, NE, USA), and UV (295-385 nm) was measured using a total UV radiometer (TUVR, The Eppley Laboratory, Newport, RI, USA).

Fruit and air temperatures were obtained using copper-constantan thermocouples attached to both the exposed and covered fruit surfaces of the sun-exposed and glass-filter treatments. Temperatures and solar radiation parameters were recorded Campbell CR10X datalogger (Campbell Scientific, Logan, UT, USA) powered by a 12V battery and solar panel.

Pigments

Chlorophyll (Chl a and Chl b) and total carotenoids (Car) were extracted from 50-150 mg of frozen exocarp tissue in 1 ml 100% acetone at –20°C for 24 h. Extracts were then centrifuged at 5,000 g for 10 min at 4°C. Absorbances were read at 470, 645, and 662 nm on a UV-visible spectrophotometer (Hewlett-Packard Company, Model 8453, Wilmington, DE, USA.). Concentrations of Chl a, Chl b, and total Car (including xanthophylls) were determined by the following equations (Lichtenthaler and Wellburn, 1983):

$$\text{Chl a} = (11.75*A_{662}) - (2.35*A_{645})$$

$$\text{Chl b} = (18.61*A_{645}) - (5.03*A_{662})$$

$$\text{Car} = [(1000*A_{470}) - (2.27*\text{Chl a}) - (81.4*\text{Chl b})]/ 227$$

Flavonoids

Flavonoids were extracted from fresh tissue using a ratio of tissue:100% methanol:hexane of 0.1:1:0.5 (w/v/v). First, powdered tissue was ground with methanol using a homogenizer (Talboys Engineering Corp., Montrose, PA, USA) for 2 min. The slurry was then transferred to an Eppendorf tube and hexane was added. After samples were centrifuged at 13,000 g for 10 min, the hexane layer was discarded and the methanol fraction was dried completely under a stream of purified N_2 gas. Samples were maintained at 2°C throughout the extraction procedure.

Dried samples were then re-constituted in mobile phase (400 μl) for measurement of naringenin by HPLC, or enzymatically hydrolyzed to measure total quercetin, kaempferol, naringenin, and naringin.

The enzymatic hydrolysis of flavonoids was performed according to the method described by Yañez and Davies (2005) with some modifications. Dried samples from the flavonoid extraction were re-suspended in 0.78 M acetate buffer (pH 4.8), 0.1 M AsA, and *Helix pomatia* Type-HP-2 β-glucuronidase (100 μl). The mixture was incubated for 17-24 h at 37°C, after which samples were centrifuged at 7,000 g for 10 min at room temperature (25°C). At this point, each sample was divided in two equal aliquots, one for naringenin, to which 25 μl of daidzein (1 mg ml^{-1}) was added as internal standard, and the other aliquot for quercetin and kaempferol detections, to which 25 μl of 7-ethoxycoumarin (1 mg ml^{-1}) was incorporated as internal standard. Standards were made at a concentration of 1 mg ml^{-1} stock solutions in methanol. They were protected from light and stored at –20°C for up to 3 months. In both aliquots, 1 ml of 100% cold acetonitrile was added and vortexed for 1 min (Vortex Genie-2, VWR Scientific, West

Chester, PA, USA). Samples were then centrifuged at 7,000 g for 5 min at room temperature (Beckman Microfuge centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA). Supernatants were transferred to new Eppendorf tubes and dried completely under a stream of purified N_2 gas. Dried samples were then reconstituted in 400 μl mobile phase for naringenin or quercetin and kaempferol assays, vortexed for 1 min, and centrifuged at 7,000 g for 5 min. Supernatants were transferred to HPLC vials. In all cases the injection volume was 150 μl.

Extracts and standards were injected into a Shimadzu HPLC system (Kyoto, Japan), consisting of a LC-10AT VP pump, a SIL-10AF auto injector, a SPD-M10A VP spectrophotometric diode array detector, and a SCL-10A system controller. Integration and collection of data was carried out using the Shimadzu EZ Start 7.1.1. SP1 software (Kyoto, Japan).

Naringenin enantiomers were separated by a Chiralcel OD-RH column (150 mm x 4.5 mm I.D., 5-mm particle size; Chiral Technologies Inc. Exton, PA, USA) under isocratic conditions at 25°C. Separation was carried out using a mobile phase of acetonitrile:water:phosphoric acid of 30:70:0.04 (v/v/v) and a flow rate of 0.4 ml min^{-1}. Naringenin enantiomers were detected at 292 nm. This stereo-selective, reverse-phase HPLC method was previously validated and described in detail by Yañez and Davies (2005).

Quercetin and kaempferol were also separated isocratically by a Chiralcel AD-RH column (150 mm x 4.5 mm I.D., 5-mm particle size; Chiral Technologies Inc. Exton, PA, USA). The mobile phase was acetonitrile:water:phosphoric acid at 42:58:0.01

(v/v/v) and a flow rate of 0.6 ml min^{-1}. Both quercetin and kaempferol were detected at 370 nm. Mobile phase solvents were filtered and degassed before use.

Statistical Analysis

The experimental design was analyzed as a split-split block with three factors: 1) genotype, 2) duration of exposure, and 3) presence or absence of UV radiation. Covered and sun-exposed sections of the fruit were also analyzed. Analysis of variance and mean separation were only performed after data met the assumption of normality, which in some cases was achieved by transforming the data using the ladder of powers ($x=y^p$). When statistical differences were found, Tukey HSD test ($p < 0.05$) was used for mean separation. Orthogonal polynomials were used to evaluate trend contrasts with a Bonferoni adjustment. These analyses were performed using the statistical package SAS Institute Inc. (Cary, NC, USA).

Flavonoid contents were quantified based on standard curves constructed using peak area ratio (PAR) against standards' concentrations. PAR was obtained by dividing peak area of the compound and peak area of the internal standard. Least squares linear regression was used for this purpose.

RESULTS

Environmental Conditions

On all dates, the glass filter (-UV) reduced UV radiation between 295 nm and 325 nm by about 95%, as well as PPFD by an average of 20% (Table 1). The surface temperatures of exposed fruit typically averaged 12°C higher than air temperature (Fig.

2). The surface temperatures of the portion of fruits that were shaded by reflective tape and the exposed surface of fruits under glass showed similar diurnal patterns. The temperatures on the exposed surface of the fruit without glass filter were around 3-4°C higher than other fruit surface temperatures during the experiments (Fig. 2).

Symptoms development

The progression of photooxidative injury was evaluated by fruit surface discoloration and changes in pigment in fruit exocarp. As duration of natural sunlight exposure increased, the fruit surface became significantly less green and more yellow measured as a decrease in hue angle (Table 2). This discoloration mainly occurred during the first 2.5 h of exposure. Fruit color also became less saturated or intense (i.e. duller) as time passed, which was indicated by a significant decrease in C values (Table 2). When genotypes were compared, both *hp-1* (*hpA*, *hpR*) mutants had the same hue or green color as the parents and the other mutants, but their color was significantly intensified (higher C) and darker (smaller L*) than their parents (Table 2). No significant differences were detected in any color parameter between fruit fully exposed to sunlight (+UV) or under the glass filter (–UV). When the effect of temperature without direct sunlight on color change was analyzed by comparing the covered and exposed sections of fruit, the exposed section had lower hue and C values, indicating greater discoloration and less color intensity, respectively (Table 3, Fig. 1C). There was also a slight lightening (larger L*) in surface color of the exposed section compared to the covered section of the fruit (Table 3). Although not statistically significant, L* showed a

negative linear trend as duration of exposure increased (Table 2), an indication of chlorophyll bleaching.

Changes in pigment concentrations in fruit exocarp confirmed the visible symptoms and color measurements. As the duration of exposure increased Chl concentrations decreased (Table 4). Chl b was degraded faster than Chl a, as represented by an increase in Chl a/Chl b ratio at 2.5 h and 5 h of sun exposure (Table 4). Total Car decreased, but only after 2.5 h of exposure (Table 4). The Car/Chl ratio increased over time, confirming the change in fruit color from green to yellow (decreasing hue angle) as duration of exposure increased (Table 2).

The glass filter that attenuated UV radiation did not significantly affect pigment composition of fruit exocarp with the exception of Chl b, which increased slightly in fruit without UV radiation (Table 4). For total Chl and Car concentrations of exposed fruit, there was a significant interaction between duration of exposure and UV radiation (Table 4). There were significant reductions in both Chl and Car concentrations in UV-exposed (+UV) fruit exocarp after 5 h of exposure (Fig. 3).

Fruit exocarp from both *hp-1* mutants (*hpA, hpR*) had significantly higher (three to five times) Chl and Car levels compared to the other genotypes (Table 4). *HpA* was the only genotype whose Car/Chl ratio was lower than its parent (Table 4). The covered section of fruit exocarp had higher Chl (a and b) and Car concentrations but lower Chl a/Chl b and Car/Chl ratios than the exposed section at both 2.5 h and 5 h of exposure (Table 3). The reduction in Chl b concentration occurred faster than that of Chl a, which was reflected by the higher Chl a/Chl b ratio of exposed fruit sections. After 5 h of exposure to natural sunlight, 37% of Chl a was lost compared to 55% of Chl b (Table 4).

Reductions in Chl b concentrations in exocarp from the exposed section of the fruit after 5 h of exposure varied with genotype (Fig. 4).

Photosynthetic Efficiency

The ratio of F_v/F_m of dark-adapted fruit did not vary among genotypes, but did with duration of exposure (Table 5). F_v/F_m or intrinsic PS II efficiency decreased substantially from 0.71 to 0.18 during the first 2.5 h of exposure to sunlight, but did not change between 2.5 h and 5 h. The absence of UV radiation had no clear effect on the apparent functionality of the fruit's photochemical apparatus. After 2.5 h of exposure, fruit under the glass filter (-UV) had a lower F_v/F_m ratio than fruit submitted to +UV, but this was reversed after 5 h of exposure (data not shown). The covered section of fruit had significantly higher F_v/F_m than the sun-exposed section both at 2.5 h and 5 h of exposure (Table 3). A significant interaction of genotype and exposure (covered or exposed) indicated that only for *B* and *Del* did the sun-exposed section of the fruit not have reduced F_v/F_m compared to the covered section of the same fruit (Fig. 5)

Flavonoids

Specific flavonoids were only studied in the most apparently tolerant genotype to photooxidative stress, *hpA*, and its parent 'Ailsa Craig' (AC). Kaempferol and quercetin concentrations were significantly higher in *hpA* fruit exocarp than AC (Table 6). No differences were found in quercetin or kaempferol concentrations, or Q/K ratio, as duration of exposure increased. The Q/K ratio is not numerically the same as the ratio of the mean concentrations of these flavonoids in the table, because the ratio is calculated

from the measured quercetin and kaempferol concentrations of individual samples. Similarly, UV radiation also did not result in significant differences in flavonoid concentrations or ratios, despite the apparently higher concentrations in -UV exocarp (Table 6 & 7).

Only the quercetin concentration was higher in the exocarp of the sunlight-exposed section compared to the covered section after 5 h of exposure (Table 3). The Q/K ratio was also significantly higher in the sunlight-exposed exocarp than in the covered exocarp after 5 h of exposure (Table 3).

The concentrations of both the R- and S-naringenin enantiomers were significantly higher in the exocarp of *hpA* mutant than in AC (Table 7). *HpA* exocarp had a larger proportion of S-naringenin than AC, represented by a lower R-/S- ratio (Table 7). Except for the lower R-/S- ratio in exocarp exposed to UV radiation, naringenin concentrations did not vary with duration of exposure or UV radiation (Table 7). No differences in naringenin concentrations between covered and exposed fruit sections were detected (data not shown).

DISCUSSION

Surface bleaching and discoloration are common symptoms of photooxidative damage of tomato fruit (Rabinowitch et al., 1974; Ramsey et al., 1952; Retig and Kedar, 1967; Tomes et al., 1956). In this study, we found that the present method of inducing photooxidative damage on detached and susceptible immature-green tomato fruit under natural conditions was effective in accelerating the development of typical sunscald symptoms. Both high temperatures and solar irradiance caused fruit surface

discoloration (Table 2), but solar irradiance was most responsible for the typical bleaching symptoms in the immature green exocarp of these tomato genotypes, which increased as duration of exposure to high irradiance increased (Table 2). Fruit discoloration from a green towards yellow color, caused by high temperature either with or without high solar irradiance was directly related to a decrease or degradation of Chl relative to Car concentrations (Table 4). This apparent greater photo-stability by Car has been previously reported in apple fruit by Merzlyak & Solovchenko (2002). Furthermore, Merzlyak, Solovchenko & Chivkunova (2002) reported that sunscald-tolerant apple cultivars (e.g. 'Zhigulevskoye) build up large amounts of Car in their sun-exposed fruit peel as a response to higher irradiance. They hypothesized that these additional Car serve as photo-protectants in these cultivars. These authors also indicated that this increase in Car does not occur in sunscald-susceptible cultivars (e.g. ‘Granny Smith’), where both Car and Chl decline in sunlight-exposed fruit peel. Car and Chl also declined in the exocarp of immature green tomato fruit in our study.

Interestingly, chlorophyll degradation and an increase in Car/Chl ratio are also part of ripening or senescence changes of many fleshy fruits, including tomatoes (Andrews *et al.*, 2004, Torres, 2001). As tomatoes ripened the Chl a/Chl b ratio in exocarp declined linearly (Andrews *et al.*, 2004), which is opposite to our results when immature green fruit developed photooxidative damage (Table 4). Similarly, an increase in chl a/ chl b ratio was reported when shaded leaves were transferred to full sunlight (Burrit & Mackenzie 2003). This response might be interesting to explore further in other fruit, such as apples, as a non-destructive method to determine the occurrence of mild photooxidative damage or sunscald.

Hp-1 (*hpA, hpR*) fruit exocarp had significantly higher Chl and Car contents compared to the other genotypes (Table 4), as has been previously reported for either exocarp or mesocarp tissues (Andrews et al., 2004; Baker and Tomes, 1964; Clayberg *et al*., 1960; Jarret *et al*., 1984; Stevens and Rick, 1986; Thompson, 1955, 1961; Torres, 2001). This characteristic of *hp-1* fruit was directly related to differences in surface color parameters. Hue angle, the parameter that represents color, was similar in all genotypes, including the two *hp-1* mutants (Table 2). However, the green coloration of the *hp-1* mutants was more saturated (lower C) and of a darker (lower L*) green color than the other genotypes (Table 2). The lower values of L* and C likely resulted from the higher Chl and Car concentrations in *hp-1* exocarp (Table 4). In agreement with preliminary studies, the *hp-1* mutants, especially *hpA*, appeared visually as the most tolerant genotype to photooxidative damage among all the genotypes that were tested. This apparent tolerance was not detectable by surface color measurement, since there was no significant interaction between genotypes and duration of exposure on L*, C, or hue angle values during the five hours of photooxidative stress (Table 2). But

Fruit exocarp of the *hp-1* mutants did not show any tolerance to photoinhibition, measured as F_v/F_m (Table 5). This suggests that their visually higher tolerance to photooxidative stress might not be a result of their higher Car content protecting the photosynthetic apparatus, but of other antioxidant components enhanced in this mutant, that is ascorbate and some enzymes involved in the ascorbate-glutathione cycle (Andrews *et al*., 2004; Jarret *et al*., 1984; Torres, 2001). These antioxidants improved the performance of the *hp-1* mutants during the imposed photooxidative stress.

High temperatures, without direct sunlight, represented by the covered section of the fruit were responsible for 53% of the decrease in photosynthetic efficiency, measured as reduction in F_v/F_m ratio in the first 2.5 h of exposure, while the presence of sunlight accounted for the remaining 22% decrease of the total of 75% decrease in F_v/F_m after 2.5 h of exposure. It has been reported that the leaves of certain desert plants have different temperature thresholds for chlorophyll fluorescence depending on their environmental adaptation. Above these temperature thresholds chlorophyll fluorescence increases drastically. This temperature threshold ranged between 42-47°C in high-temperature adapted species (Seemann, Berry & Downton 1984). Ludlow & Björkman (1984) also described a similar phenomenon in *Macroptilium atropurpureum* 'Siratro' leaves at temperatures over 42°C under high light conditions, which they called "high-temperature-induced photoinhibition". In our study, thermo-inhibition was detected after 2.5 h of sun exposure when fruit surface temperatures averaged 46°C (Fig. 1). It is also possible that this high-temperature effect occurred prior to 2.5 h of sun-exposure at lower surface temperatures, especially since Smillie, Hetherington & Davies (1999) showed that PSII efficiency decreased more rapidly in fruit than in leaves with increasing PPFD. Thermo-inhibition as well as photoinhibition could cause increases in ROS flux in the cell and possible up-regulation of antioxidant systems comprised by metabolites and enzymes to cope with the increased ROS.

There were no detectable changes in concentrations of quercetin, kaempferol, or naringenin enantiomers in fruit exocarp as duration of exposure increased (Table 5, 6 & 7), yet kaempferol and especially quercetin accumulated rapidly when fruit was exposed to sunlight (Table 3). These results suggest that kaempferol and quercetin synthesis

might be triggered as a photo-protective mechanism to a sudden increase in light levels as suggested by Close & McArthur (2002) for leaves and Merzlyak *et al*. (2002) in apple fruit peel. However, once the synthesis of these flavonoids is induced their concentrations under the same light conditions might stay constant or increase at a much slower rate. Interestingly, the concentration of naringenin did not respond to the presence or absence of sunlight (data not shown).

The presence or absence of natural UV light did not have an effect on flavonoid accumulation (Table 5, 6 & 7), as has been shown in previous reports on leaves by Ryan, Swinny, Markham & Winefield (2002). This suggests that the accumulation of flavonoids as a photo-protective mechanism might be rapidly engaged by a sudden increase of visible, but not UV light. It is also possible that UV-B induction of flavonoids, which has been shown to occur (Ryan *et al*. 2002; Solovchenko & Schmitz-Eiberger 2003), is a slower process under natural sunlight. It is important to mention that phenolics are able not only to absorb UV-B radiation *in vitro*, but also visible radiation (Jordan 1996), minimizing photo-damage suffered by the photosynthetic apparatus under high light conditions.

The rapid accumulation of quercetin in exposed sections of fruit exocarp resulted in a significantly higher Q/K ratio after 5 h of exposure (Table 3). Curiously, although quercetin and kaempferol did not increase in the +UV treatment, the Q/K ratio did (Table 6). According to the observations of Ryan *et al*. (2002), an increase in Q/K ratio might be a photo-protective mechanism that occurs with higher levels of UV-B radiation. This supposition is based on the fact that quercetin (an *ortho*-dihydroxylated flavonoid) showed higher antioxidant capacity in vitro than its mono-hydroxylated flavonoid

equivalent, kaempferol (Montesinos, Ubeda, Terencio, Paya & Alcaraz 1995), and so, quercetin synthesis is favored over kaempferol under light-stress conditions. However, an increasing Q/K ratio could be merely due to faster rate of degradation of kaempferol than quercetin in high sunlight.

Previous reports on *hp-1* mutants have indicated the enhancement of anthocyanin levels in several plant parts (Jarret *et al*. 1984; Wettstein-Knowles 1968a). In our study, we found that all flavonoids analyzed were greatly enhanced in *hpA* fruit exocarp compared to its parent, AC (Tables 6 & 7). This is another pleitropic effect of the mutation on the HP genes, which are believed to be negative regulator(s) of phytochrome signal translation (Kerr 1965; Kerckhoffs *et al*. 1997; Mustilli, Fenzi, Ciliento, Alfano & Bowler 1999; Peters *et al*. 1998).

In the present study, we have been able to successfully follow the progression of photooxidative stress in detached fruit under natural solar irradiance. Using this system, we have identified factors involved in the development of visual symptoms of sunscald and damage to the photosynthetic apparatus during photooxidative stress episodes. We have been also able to determine, that as well as in leaves, flavonoids are rapidly induced in fruit exocarp exposed to high light as a physiological acclimation response. However, there was insufficient evidence to indicate whether UV light itself plays a role in sunscald development. Surprisingly, it does not appear that the natural UV radiation levels at our experimental site were responsible for the increase in flavonoids in the fruit of any of the genotypes studied.

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LEGENDS

Figure 1. Experimental setup under glass filter (A), individual immature green fruit covered with reflective tape (B), and the appearance of the exposed and covered sections of fruit after 2.5 h of exposure to natural sunlight.

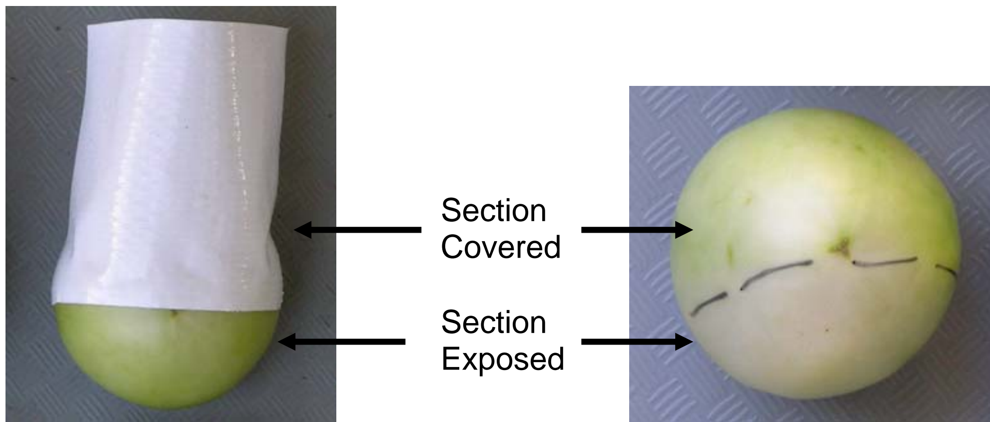
Figure 2. Temperature pattern during a typical experimental day (29 July 2003), with treatments of covered fruit surface under glass filter (-▼-), exposed fruit surface under glass filter (-▽-), covered fruit surface without glass (-■-), exposed fruit surface without glass (-□-), air (-◆-), and air under glass filter (-◇-).

Figure 3. Effect of duration of exposure (2.5 h and 5.0 h) and UV radiation on total chlorophyll (Chl) and carotenoid (Car) concentrations in sunlight-exposed fruit exocarp from immature green fruit. Different upper- and lower-case letters indicate statistical differences (Tukey, HSD, $P<0.05$) for Chl and Car, respectively.

Figure 4. Effect of genotype and exposure (covered or exposed) on chlorophyll (Chl) b concentration in fruit exocarp from immature green fruit after 5 h of exposure to sunlight. (*) indicates statistically different covered versus exposed within genotypes (Tukey, HSD, $P<0.05$).

Figure 5. Effect of genotype and exposure (covered or exposed) on Fv/Fm ratio in fruit exocarp from immature green fruit after 2.5 h of exposure to sunlight. (*) indicates statistically differences between covered and exposed within genotypes (Tukey, HSD, $P<0.05$).

(A)



(B) (C)

Figure 1

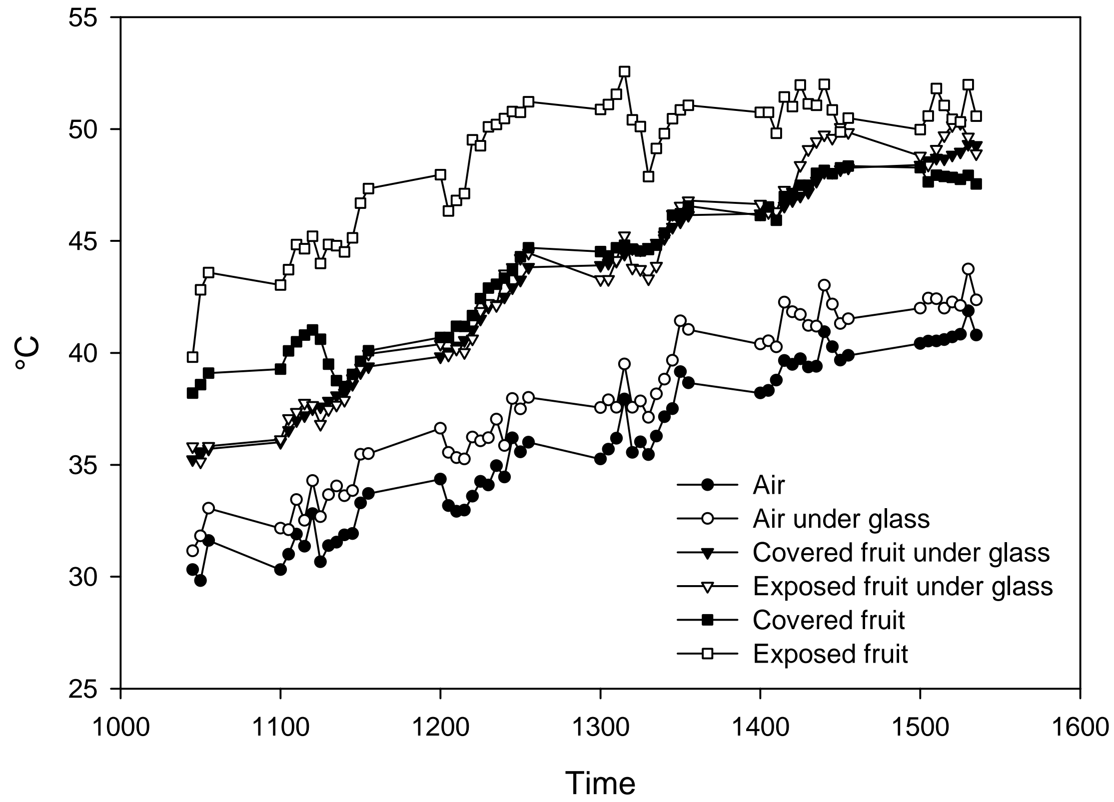


Figure 2

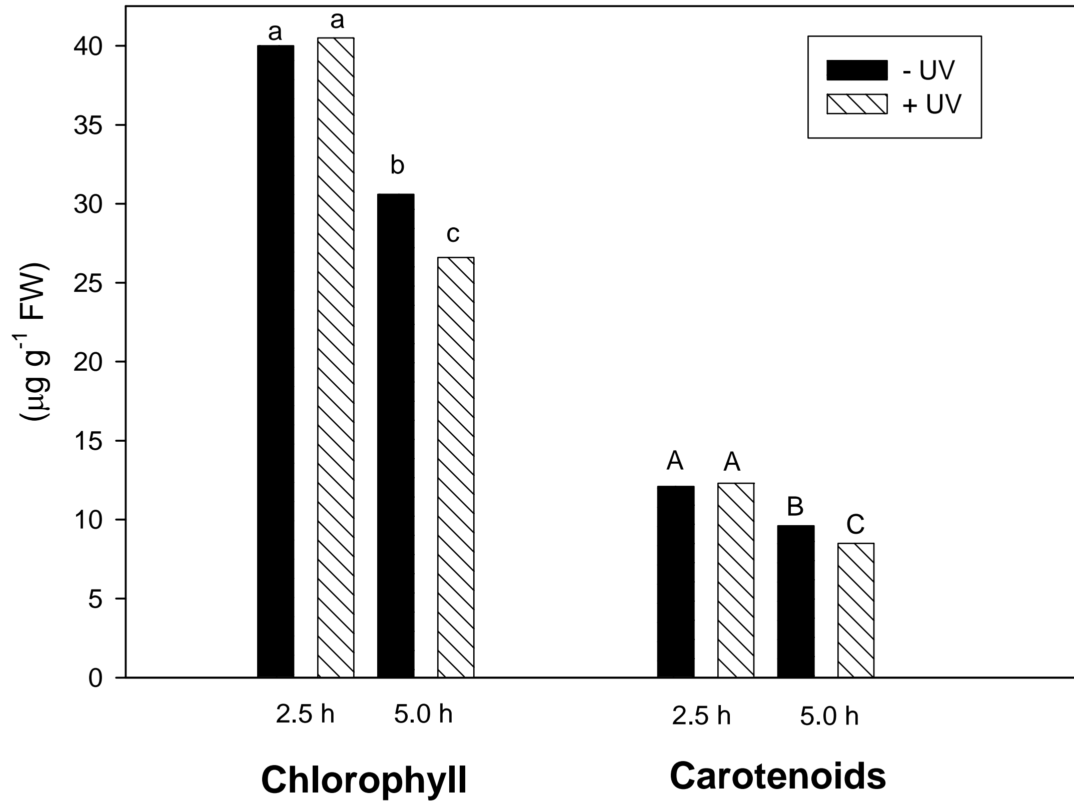


Figure 3

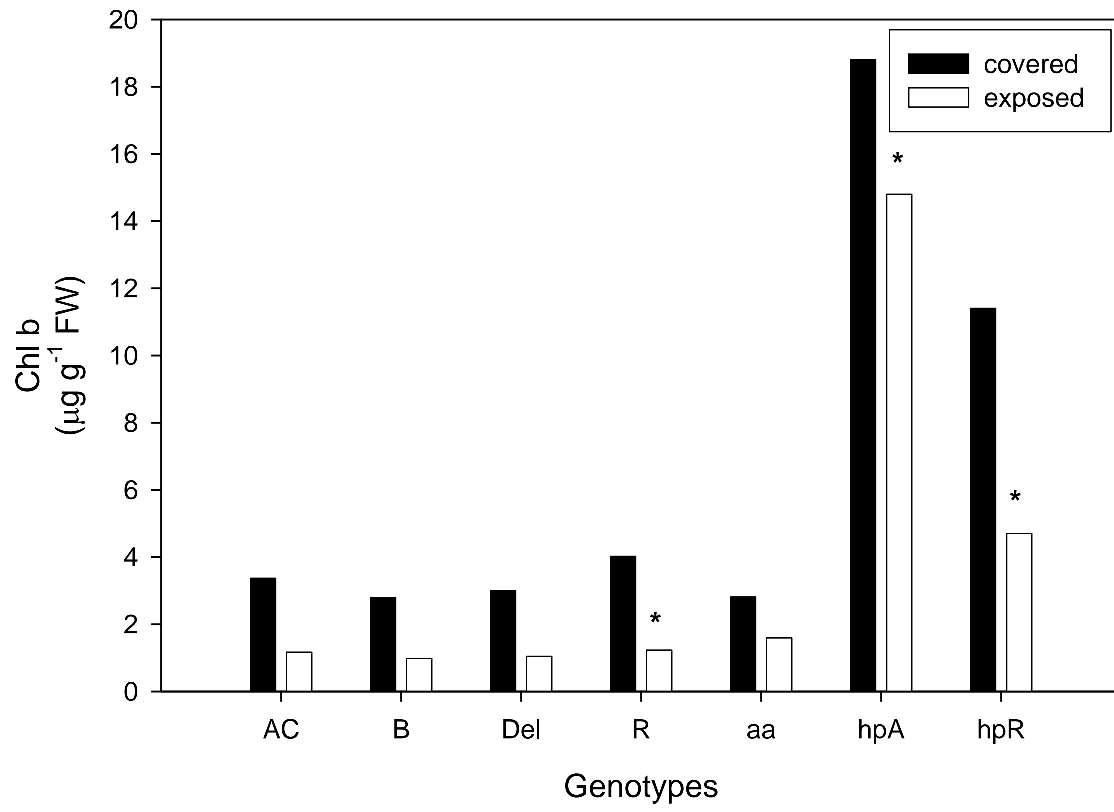


Figure 4

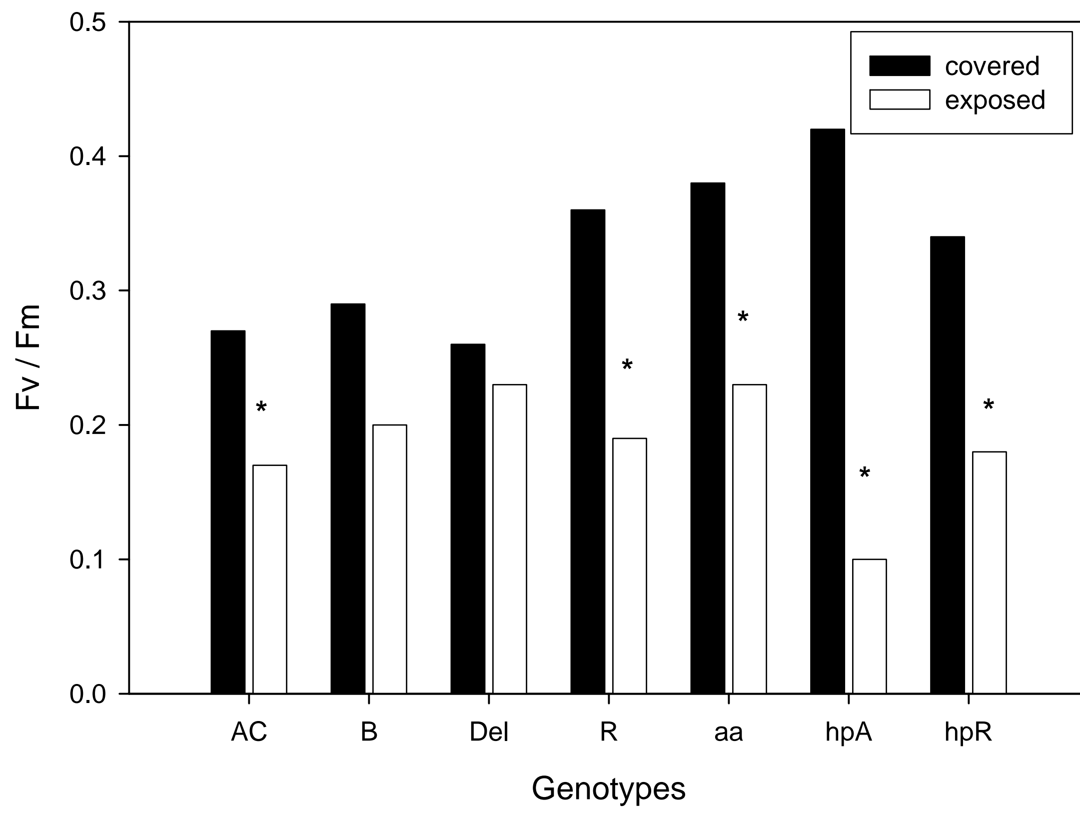


Figure 5

Table 1. Average and cumulative values from 11:00 am to 5:00 pm Pacific Daylight Savings Time for total radiation, photosynthetic photon flux density (PPFD), and ultraviolet (UV) radiation (295 – 325 nm) on all experimental dates in 2003.

Dates	17 July		22 July		29 July		13 August		26 August	
	-UV	+UV	-UV	+UV	-UV	+UV	-UV	+UV	-UV	+UV
Radiation (W m^{-2})										
Average		975		1020		994		956		650
Cumulative		57808		65720		64820		57385		37290
PPFD (μmol m^{-2} s^{-1})										
Average	1228	1430	1672	1316	1281	1787	1171	1400	990	1291
Cumulative	72935	84180	78988	100337	76854	101246	70240	84020	59310	77447
UV (W m^{-2})										
Average	0.14	3.11	0.17	4.05	0.20	3.76	0.08	3.39	0.02	2.83

Table 2. Surface color parameters for lightness (L*), chroma (C), and hue angle from exocarp of immature green fruit of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (UV) radiation (C) on sections of exposed fruit. P values indicated for comparisons within columns. Trend contrasts P values represent a Bonferoni adjustment.

Factors	L*	C	Hue (°)
Genotypes (A)			
'Ailsa Craig'	65.2 ab[Y]	32.4 ac	110.7
Aa	67.3 a	29.6 ab	110.4
B	67.3 a	29.0 ab	109.8
Del	65.7 a	31.7 ac	110.0
hpA	58.6 c	37.1 d	111.7
'Rutgers'	66.3 a	26.9 b	110.9
hpR	61.9 bc	35.7 cd	110.7
P value	<.0001	<.0001	0.481
Duration (h) (B)			
0	65.2	33.8	114.3
2.5	64.8	31.4	109.4
5.0	63.9	30.1	108.0
P value - Linear	0.140	0.000	<.0001
P value – Non-linear	0.954	0.466	0.003
(A) x (B) P value	0.719	0.984	0.874
UV radiation (C)			
+ UV	64.5	31.4	110.4
- UV	64.7	32.1	110.8
P value	0.508	0.109	0.105
(A) x (C) P value	0.259	0.920	0.370
(B) x (C) P value	0.512	0.175	0.237
(A) x (B) x (C) P value	0.489	0.715	0.760

[Y] Different letters within columns indicate statistical differences. Protected LSD($P<0.05$).

Table 3. Surface color parameters (lightness, L*; chroma, C; and hue angle), pigment concentrations (chlorophyll; Chl a, Chl b, total Chl; carotenoids, Car), fluorescence parameters (F_v/F_m), and flavonoids (kaempferol, K; quercetin, Q; Q/K ratio) from sunlight exposed and covered sections from immature green fruit. P value indicated for comparison within rows.

Variable	Duration of exposure (h)	Exposed	Covered	P value
Color Parameters				
L*	2.5	64.8	64.2	0.004
	5.0	63.9	63.3	0.073
C	2.5	31.4	33.6	<.0001
	5.0	30.1	34.1	<.0001
Hue(°)	2.5	109.4	111.7	<.0001
	5.0	108.1	111.2	<.0001
Pigments				
Chl a	2.5	36.4	40.3	<.0001
($\mu g\ g^{-1}$ FW)	5.0	24.4	35.7	<.0001
Chl b	2.5	5.0	7.7	<.0001
($\mu g\ g^{-1}$ FW)	5.0	3.4	6.8	<.0001
Tot. Chl	2.5	41.4	48.0	<.0001
($\mu g\ g^{-1}$ FW)	5.0	27.7	42.5	<.0001
Car	2.5	12.5	12.9	<.0001
($\mu g\ g^{-1}$ FW)	5.0	8.9	11.8	0.006
Chl a/Chl b	2.5	12.9	6.9	0.002
	5.0	9.2	6.1	<.0001
Car / Chl	2.5	0.25	0.31	<.0001
	5.0	0.38	0.31	0.001
Chl Fluorescence				
F_v/F_m	2.5	0.18	0.33	<.0001
	5.0	0.19	0.31	0.001
Flavonoids				
K	2.5	233.3	209.7	0.598
($\mu g\ g^{-1}$ FW)	5.0	236.5	201.7	0.115
Q	2.5	1501.9	1315.9	0.192
($\mu g\ g^{-1}$ FW)	5.0	3051.2	1268.2	0.032
Q / K	2.5	6.3	6.3	0.929
	5.0	12.3	5.9	0.024

Table 4. Chlorophyll (Chl) a, Chl b, total Chl, and total carotenoid (Car) concentrations from exocarp of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (UV) radiation (C) on sections of exposed immature green fruit. P values indicated for comparisons within columns. Trend contrasts P values represent a Bonferoni adjustment.

Factors	Chl a ($\mu g\ g^{-1}$ FW)	Chl b ($\mu g\ g^{-1}$ FW)	Total Chl ($\mu g\ g^{-1}$ FW)	Total Car ($\mu g\ g^{-1}$ FW)	Chl a/Chl b	Car / Chl
Genotypes (A)						
'Ailsa Craig'	17.3 a[Z]	2.60 a	19.9 a	6.30 a	10.0	0.34 ab
aa	16.6 a	2.55 a	19.2 a	6.08 a	11.5	0.34 abc
B	15.4 a	1.64 a	17.0 a	5.75 a	10.2	0.38 a
Del	17.6 a	2.50 a	20.1 a	6.71 a	8.9	0.36 a
hpA	100.7 c	19.16 c	119.9 c	30.08 c	5.4	0.26 c
'Rutgers'	10.8 a	1.77 a	12.6 a	3.82 a	10.3	0.32 ab
hpR	57.8 b	7.77 b	65.6 b	17.15 b	8.5	0.29 bc
P value	<.0001	<.0001	<.0001	<.0001	0.1482	0.001
Duration (h) (B)						
0	39.1	7.57	46.6	11.1	5.5	0.25
2.5	36.4	5.00	41.4	12.5	12.9	0.35
5.0	24.4	3.37	27.7	8.9	9.2	0.38
P value - Linear	<.0001	<.0001	<.0001	0.000	0.005	<.0001
P value – Non-linear	0.360	0.290	0.710	0.000	<.0001	0.005
(A) x (B) P value	0.238	0.519	0.175	0.252	0.587	0.139
UV radiation (C)						
+ UV	29.5	3.94	33.5	10.4	10.3	0.41
- UV	30.9	4.41	35.3	10.8	11.6	0.35
P value	0.544	0.022	0.091	0.086	0.943	0.074
(A) x (C) P value	0.455	0.408	0.426	0.167	0.525	0.069
(B) x (C) P value	0.277	0.055	0.018	0.021	0.036	0.779
(A)x(B)x(C) P value	0.332	0.201	0.061	0.170	0.422	0.058

[Z] Different letters within columns indicate statistical differences. Protected LSD(P<0.05).

Table 5. F_v/F_m ratio of immature green fruit surface of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (UV) radiation (C) on sections of exposed fruit. P values indicated for comparisons within columns. Trend contrasts P values represent a Bonferoni adjustment.

Factors	F_v/F_m
Genotypes (A)	
'Ailsa Craig'	0.34
aa	0.40
B	0.36
Del	0.38
hpA	0.34
'Rutgers'	0.37
hpR	0.38
P value	0.282
Duration (h) (B)	
0	0.71
2.5	0.18
5.0	0.19
P value - Linear	<.0001
P value – Non-linear	<.0001
(A) x (B) P value	0.389
UV radiation (C)	
+ UV	0.37
- UV	0.37
P value	0.896
(A) x (C) P value	0.206
(B) x (C) P value	0.012
(A) x (B) x (C) P value	0.210

Table 6. Total kaempferol (K) and quercetin (Q) concentrations and Q/K ratio from exocarp of immature green fruit from 'Ailsa Craig' and *hpA* (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (UV) radiation (C) on sections of exposed fruit. P values indicated for comparisons within columns. Trend contrasts P values represent a Bonferoni adjustment.

Factors	Kaempferol ($\mu g\ g^{-1}$ FW)	Quercetin ($\mu g\ g^{-1}$ FW)	Q/K
Genotypes (A)			
'Ailsa Craig'	185.7	831.9	5.2
hpA	277.7	4280.2	14.9
P value	0.042	0.033	0.059
Duration (h) (B)			
0	225.3	3345.7	11.9
2.5	233.3	1599.3	6.6
5.0	236.5	3051.2	12.3
P value - Linear	0.479	0.224	0.081
P value – Non-linear	0.345	0.143	0.321
(A) x (B) P value	0.459	0.215	0.432
UV radiation (C)			
+ UV	190.9	1753.9	10.6
- UV	257.1	2529.1	7.9
P value	0.192	0.943	0.071
(A) x (C) P value	0.051	0.489	0.184
(B) x (C) P value	0.078	0.914	0.192
(A) x (B) x (C) P value	0.227	0.737	0.188

Table 7. R-naringenin, S-naringenin, total naringenin concentrations and R-naringenin/S-naringenin ratio (R-/S-) from exocarp of immature green fruit from 'Ailsa Craig' and *hpA* (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (UV) radiation (C) on sections of exposed fruit. P values indicated for comparisons within columns. Trend contrasts P values represent a Bonferoni adjustment.

Factors	R-naringenin (μg g^{-1} FW)	S-naringenin (μg g^{-1} FW)	Total naringenin (μg g^{-1} FW)	R-/S-
Genotypes (A)				
'Ailsa Craig'	53.4	59.8	113.2	0.91
hpA	75.0	140.1	215.1	0.56
P value	0.056	0.010	0.010	0.001
Duration (h) (B)				
0	54.5	91.6	146.1	0.66
2.5	73.2	116.0	188.2	0.73
5.0	65.2	92.6	157.8	0.82
P value - Linear	0.213	0.245	0.165	0.216
P value – Non-linear	0.198	0.132	0.213	0.199
(A) x (B) P value	0.627	0.367	0.450	0.356
UV radiation (C)				
+ UV	62.6	101.9	164.5	0.67
- UV	71.2	108.0	182.2	0.77
P value	0.437	0.972	0.593	0.017
(A) x (C) P value	0.069	0.252	0.298	0.301
(B) x (C) P value	0.324	0.073	0.165	0.191
(A) x (B) x (C) P value	0.860	0.679	0.725	0.738

CHAPTER 3

BIOCHEMICAL RESPONSES OF FRUIT EXOCARP OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL) MUTANTS TO NATURAL PHOTOOXIDATIVE CONDITIONS

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ABSTRACT

Photooxidative damage in fruit is caused by high solar irradiance and temperatures, resulting in increased reactive oxygen species. Using a system that permitted the imposition of photooxidative stress under natural solar radiation, we evaluated the changes in antioxidant metabolites and enzymes, and damage to macromolecules, in exocarp of immature green tomato (*Lycopersicon esculentum* Mill) fruit. Exposed or covered sections of detached fruit of mutants (*anthocyanin absent*, *β-carotene*, *Delta*, and *high pigment-1, hp-1*) with attenuated pigment and/or antioxidant metabolite levels, and their nearly isogenic parents ('Ailsa Craig' and 'Rutgers'), were subjected to five hours of high solar irradiance, either in the presence or absence of ultraviolet (UV) radiation. Ascorbate and glutathione pools and total soluble protein decreased in fruit exocarp as duration of exposure to photooxidative stress increased. Specific enzyme activities of superoxide dismutase, ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and catalase (CAT) increased with duration of exposure, suggesting that these proteins were conserved during stress exposure. UV radiation contributed to increased APX and MDHAR specific activities. Covered sections of fruit had more protein and higher APX but lower CAT activities than exposed exocarp. The *hp-1* mutant had higher ascorbate and protein concentrations and APX and GR activities than the other genotypes. Either because of the sufficiency of the antioxidant systems in these genotypes or the short duration of the photooxidative stress, there were no effects on lipid peroxidation or nuclease and proteinase activities.

Key words: acclimation, antioxidant, oxidative, photooxidation, stress, sunscald

INTRODUCTION

Immature green fleshy fruits can be subjected to light-mediated tissue deterioration from photooxidative stress, leading to sunscald (Barber & Sharpe 1971; Retig & Kedar 1967; Walker 1957). This damage is the result of photodynamic injury of heated tissue, which occurs under intense solar irradiance and results in tissue discoloration and bleaching of affected sun-exposed fruit surfaces (Barber & Sharpe 1971; Brooks & Fisher 1926). These symptoms are believed to occur from damage caused by reactive-oxygen-species (ROS), the flux of which is greatly increased under photooxidative stress (Foyer, Descourvieres & Kunert 1994).

ROS are produced normally and continuously by cells, yet they are tightly controlled by complex and dynamic antioxidant systems within cells. Plant antioxidant defenses are comprised of both enzymes and non-enzymatic metabolites that are localized in specific tissues (e.g., epidermis/hypodermis) and cellular compartments (e.g., chloroplasts), which function in a complex series of overlapping oxidation-reduction pathways. For example, the various isoenzymes of superoxide dismutase (SOD; EC 1.15.1.1) catalyze the dismutation of the superoxide anion ($O_2^{\cdot -}$), producing hydrogen peroxide (H_2O_2), which in turn, is reduced to water by either catalases (CAT; EC 1.11.1.6) (Willekens, Chamnongppol, Davey, Schraudner, Langebartels, Van Montagu, Inzé & Van Camp 1995) or ascorbate peroxidases (APX; EC 1.11.1.11). Chloroplasts contain stromal, thylakoid-bound, and lumenal forms of APX (Asada 1999). APXs use reduced ascorbic acid (AsA) as an electron donor (Groden & Beck 1979, Anderson, Foyer & Walker 1983). The oxidized form of AsA, monodehydroascorbate

(MDHA), is reduced to AsA via reduced ferredoxin, generated as a result of photosynthetic electron flow (Miyake & Asada 1994).

Chloroplasts contain another pathway involving AsA, the ascorbate-glutathione cycle (Foyer & Halliwell 1976), which removes H_2O_2 and consumes reducing power [NAD(P)H] while producing ATP. In this cycle, MDHA generated via the APX reaction is converted back to AsA via monodehydroascrobate reductase (MDHAR; EC 1.6.5.4). Any MDHA that escapes this route of re-cycling rapidly disproportionates to dehydroascorabate (DHA) and AsA (Foyer *et al*. 1994). DHA is converted back to AsA by the action of dehydroascorabate reductases (DHAR; EC 1.8.5.1). DHAR utilizes reduced glutathione (GSH), the predominant non-protein thiol in plants (Rennenberg 1982), as reductant, GSH is regenerated by glutathione reducatse (GR; EC 1.6.4.2) from its oxidized form, glutathione disulphide (GSSG). Catalase (CAT), found primarily in peroxisomes, catalyzes the dismutation of H_2O_2 at high concentrations produced by photorespiration into water and oxygen (Schonbaum & Chance 1976).

Among fruit crops, tomatoes (*Lycopersicon* spp.) have long served as model species for understanding fruit physiology, biochemistry, and genetics. In the present study we selected tomato mutants with attenuated pigments and/or antioxidant metabolites to study photooxidative stress in fruit. We implemented a system utilizing detached fruit and natural solar radiation. In the first part of this study we present results and discussion concerning physiological factors involved in photooxidative stress and sunscald development (Chapter 2). In this second part of the study, we present biochemical data associated with photooxidative stress and sunscald development under natural solar radiation and discuss the role that different components of

antioxidant systems, both metabolites and enzymes, could play in fruit acclimation to photooxidative stress.

MATERIALS AND METHODS

Tomato mutants

The tomato (*Lycopersicon esculentum* Mill.) genotypes selected for this study were: 1) *anthocyanin absent* (*aa*) (LA 3617), 2) *β-carotene* (*B*) (LA 3179), 3) *Delta* (*Del*) (LA2996A), 4) *high pigment* (*hp-1*) (LA2838A, *hpA* and LA3004, *hpR*). All mutants are nearly isogenic in the cultivar "Alisa Craig' (AC), except LA3004, which is nearly isogenic in 'Rutgers' (R). Full descriptions of each tomato mutant are given in Chapter 2.

Seeds from these previously described tomato mutants and their parents were obtained from the C.M. Rick Tomato Genetics Resource Center (University of California, Davis, CA).

Field site

The research site was located in Lewiston, Idaho (46°23' N; 116°59' W) at an elevation of 430 m above sea level. The site was level and had a uniform soil of Nez Perce silty, clay-loam texture (fine, montmorillonitic, mesic Xeric Argialbolls). This region is classified as desert steppe with summer (June-Sept.) mean maximum temperatures of 30°C on generally cloudless days, as most precipitation occurs only in the winter months.

Seedlings of the tomato genotypes were started in cell packs in a greenhouse until they had several true leaves, at which time they were transplanted in a replicated, randomized complete block design in the field, consisting of 4 blocks with six plants per plot. Plants were spaced 1.2 m apart in rows, with rows 2.4 m apart.

All plants were irrigated with buried drip tape, one hour per day during the first 30 days after planting, and two hours per day from 30 days after planting until the end of the growing season. The output of each emitter was 1.9 L hr^{-1}. Plants were surface fertilized two weeks after transplanting with calcium nitrate (30 g per plant).

Experimental Setup

Determination of susceptibility to photooxidative damage was evaluated by exposing detached immature green fruit from all genotypes to treatments of natural sunlight, either with or without exposure to ultraviolet (UV) radiation (+UV or –UV, by the use of 0.635 cm thick safety glass plate filter), for three time durations of exposure (i.e. 0, 2.5, and 5 h). The glass plate filtered 95% of UV radiation (295 – 325 nm). In order to investigate the independent effects of solar radiation and high temperature on photooxidative injury, each fruit from the treatments just described was partially covered with reflective tape allowing air movement on the fruit surface beneath (see Chapter 2, Fig. 1).

Each treatment was represented by a group of four similar-sized fruits, harvested from non-sunlight exposed locations within the plant canopies just before setting up the experiment, for a total of 168 fruit tested per date. Harvested fruits were placed on top of a white board with the calyx-end up. These sets of treatments were replicated on five

separate dates in 2003: 17, 22, and 29 July and 13 and 26 Aug. On all dates, experiments were conducted between 11:30 a.m. and 5:00 p.m. Pacific Daylight Savings time. Each date was considered a block.

Sampling

The exocarp (up to 2 mm thick) of fruit submitted to the treatments was removed using a scalpel, and separately from the two sections, exposed and covered with reflective tape. Only light green tissue was sampled (no dark green or “green shoulder” tissue) from immature green fruit to maintain sampling homogeneity. Samples were immediately frozen in liquid N_2, transported on dry ice, and stored at –80°C for later analysis. Before biochemical measurements, samples were finely ground in liquid N_2 with a mortar and pestle, and weighed for each of the different assays.

Extraction and Assay of Metabolites

Approximately 0.2 g of exocarp tissue per sample was extracted by grinding in a mortar and pestle with liquid N_2, acid-washed sand, and 1.5 ml of 1 M $HClO_4$ as extractant. Following centrifugation at 13,000 g for 10 min at 4°C, supernatants were partitioned into two, 400 μl aliquots for ascorbic acid (AsA) and glutathione (GSH) determinations.

To these extracts, 200 and 100 μl of 0.1 M HEPES/KOH buffer (pH 7.0) was added for AsA and GSH determinations, respectively. Aliquots of 6 M K_2CO_3 were incorporated gradually to adjust pH to 4.0-5.0 for AsA determination or 6.0-7.0 for GSH

determination, and to precipitate perchlorate. Samples were centrifuged at 13,000 g for 10 min at 4°C and the pellets were discarded.

Reduced AsA and oxidized DHA were assayed spectrophotometrically via a kinetic reaction at 265 nm by adding 4 units ascorbate oxidase (from *Cucurbita* sp, Sigma-Aldrich, USA) to a 0.5 ml reaction mixture containing 0.1 M Na_2HPO_4 (pH 5.6) and 10 μl sample extract (Andrews, Fahy & Foyer 2004). For DHA determination, 100 μl extract was incubated for 5 min on ice with 50 mM dithiothreitol (DTT) in 0.1 M Na_2HPO_4 (pH 7.5) buffer. Reduced glutathione (GSH) and its oxidized form (GSSG) were measured spectrophotometrically by methods described by Owen & Belcher (1965), Tietze (1969), and Griffiths (1980) via a kinetic reaction at 412 nm by adding 1.0 unit glutathione reductase (from Bakers yeast, Sigma- Aldrich, USA) to a 1 ml reaction mixture containing 0.1 M Na_2HPO_4 (pH 7.5) buffer, 6 mM EDTA, 6 mM 5-5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 10 mM NADPH, and 10 μl sample extract.

Extraction and Assay of Enzymes

Similar extraction procedures were used for enzymes as described previously for the antioxidant metabolites, except that the extractant contained 50 mM MES/KOH (pH 6.0) buffer, 40 mM KCl, 2 mM $CaCl_2$, and 1 mM L-AsA. After centrifugation at 13,000 g for 10 min at 4°C, supernatants were used immediately for enzyme activity assays, except SOD, for which an aliquot of supernatant was stored at –80°C for later assay. Bradford's (1976) method was used to determine soluble protein content in the sample. All enzyme activity assays were conducted at 20°C in 0.5 ml reaction volume.

SOD activity was assayed as described by McCord & Fridorich (1969) with some modifications. The reaction mixture consisted in 50 mM HEPES (pH 7.8) buffer, 0.5 mM EDTA; 0.5 mM nitro-blue tetrazolium; 4 mM xanthine; 50 μl extract; and 0.04 unit xanthine oxidase. After 10 min, absorbance was measured at 560 nm. SOD activity was determined by a standard curved constructed using horseradish SOD (Sigma-Aldrich, St. Louis, MO, USA).

APX activity was assayed by a procedure of Nakano & Asada (1987) with modifications. In a reaction mixture containing 50 mM KH_2PO_4 (pH 7.0) buffer, 250 μM L-AsA, and 10 μl extract, 5 mM H_2O_2 was added to initiate the reaction. Change in absorbance was monitored at 290 nm for 3 min and activity was calculated from this reaction rate, using an extinction coefficient of 2.8 mM^{-1}.

MDHAR activity was measured in an assay mixture containing 100 mM HEPES (pH 7.6) buffer, 2.5 mM L-AsA, 250 μM NADH, and 10μl extract with 0.4 unit of ascorbate oxidase added to start the reaction. Change in absorbance was monitored at 340 nm for 3 min and activity was calculated from this reaction rate using an extinction coefficient of 3.3 mM^{-1} (Miyake & Asada 1992).

DHAR activity was determined according to Miyake & Asada (1992). In a reaction mixture containing 50 mM HEPES (pH 7.0) buffer, 0.1 mM EDTA, 2.5 mM GSH, and 10 μl extract, 0.2 mM DHA was added to initiate the reaction. Change in absorbance was measured at 265 nm for 3 min and activity was calculated from this reaction rate using an extinction coefficient of 7.0 mM^{-1}.

GR was measured by the method of Foyer & Halliwell (1976). The assay mixture contained 50 mM HEPES (pH 8.0) buffer, 0.5 mM EDTA, 250 μM NADPH, and 10μl

extract, with 500 μM GSSG added to start the reaction. Change in absorbance was monitored at 340nm for 3 min. Prior to this measurement non-enzymatic NADPH oxidation was measured in each reaction mixture before GSSG was added. Enzyme activity was calculated by subtracting the rate of the non-enzymatic reaction from the rate of the GR-specific activity using an extinction coefficient of 6.22 mM^{-1}.

CAT was measured spectrophotometrically using the method of Chance & Maehly (1955) in a reaction mixture containing 50 mM KH_2PO_4 (pH 7.0) buffer, 15 mM H_2O_2, and 100 μl extract to initiate the reaction. Activity was expressed as the change in absorbance at 240 nm as 50 mM H_2O_2 was degraded. Catalase activity was calculated using an extinction coefficient of 39.4 mM^{-1} (Aebi, 1983).

Nuclease activity was measured spectrophotometrically based on the method of Blank & McKeon (1989) and modified by Hosseini & Mulligan (2002). Samples were run in duplicates with reaction mixtures containing 0.02% (w/v) single-stranded calf-thymus DNA (Sigma-Aldrich, St. Louis, MO, USA), 0.01% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA), 25 μL extract, and 440 μL of buffer containing 0.05 M Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 μM $ZnCl_2$. One of the duplicate assays was stopped with 3.5% (v/v) perchloric acid immediately after adding DNA, and the other duplicate assay was similarly terminated 20 min later. After centrifugation at 7,000 g for 5 min at 25°C, the absorbances of both duplicates were measured at 260 nm. Nuclease activity was expressed as change in A_{260} (due to nucleotides released from single-stranded DNA) mg $protein^{-1}$ min^{-1}.

Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA), a secondary product in the oxidation of polyunsaturated fatty acids, by the modified thiobarbituric acid-reactive-substances (TBARS) assay proposed by Hodges, DeLong, Forney & Prange (1999). One gram of tissue was homogenized in 25 ml 80:20 (v/v) ethanol:water and centrifuged at 7,000 g for 10 min at room temperature (25°C). One ml of supernatant was taken and added to a –TBA solution [20% (w/v) trichloroacetic acid + 0.01% butylated hydroxytolueno] or a +TBA solution [20% (w/v) trichloroacetic acid + 0.01% butylated hydroxytolueno + 0.65% thiobarbituric acid, TBA]. After mixing, the samples were heated at 95°C for 25 min, cooled, and centrifuged at 7,000 g for 10 min. Absorbances at 440 and 532 nm were measured spectrophotometrically (Hewlett-Packard Company, Model 8453, Wilmington, DE). MDA equivalents were calculated as follows:

$$A = (A_{532\ +TBA} - A_{600\ +TBA}) - (A_{532\ -TBA} - A_{600\ -TBA})$$

$$B = (A_{440\ +TBA} - A_{600\ +TBA})\ 0.0571$$

$$\text{MDA equivalents (nmol ml}^{-1}) = ((A\text{-}B) / 157000) * 10^{6}.$$

Nuclease and Proteinase Activity Gels

Nucleases activity gels were performed by the method described by Blank & McKeon (1991) with some modifications. Sample extracts were boiled for 10 min in buffer consisting of 0.1 M Tris-HCl (pH 7.4) and 1.5% sodium dodecyl sulfate (SDS), and then centrifuged at 7,000 g for 5 min at 25°C. Samples equalized by fresh weight (0.25 mg FW) were then mixed with loading buffer containing 50 mM Tris-HCl (pH 6.8),

2% (w/v) SDS, 0.02% (w/v) bromophenol blue, and 10% (v/v) glycerol, and then loaded on to activity gels. Protein separation was performed using one-dimensional, discontinuous, 10% (w/v) polyacrylamide, 0.1% SDS (SDS-PAGE; Laemmli 1970) gel system with the addition of 50 $\mu g\ ml^{-1}$ single-stranded calf-thymus DNA (Sigma-Aldrich, St. Louis, MO, USA) to the resolving gel. Gels were run at 120V for 2 h at 25°C, after which nucleases were renatured by immersing the gels in renaturation buffer consisting of 0.1 M Tris-HCl (pH 7.4), 1% (w/v) Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 μM $ZnCl_2$) with shaking for 1 h at 37°C. After washing twice with 0.1 M Tris-HCl (pH 7.4) rinsing buffer, gels were incubated in 0.05 M Tris-HCl (pH 7.5) developing buffer, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 μM $ZnCl_2$ at 37°C for 12 h. Nuclease activity bands were visualized after "negative staining" the gels with 0.1% (w/v) toluidine blue for 1 h with gentle shaking. Digested substrate appeared as colorless bands. Molecular weights were determined by comparing nuclease mobility with a pre-stained molecular weight protein ladder (Invitrogen Co.,Carlsbad, CA).

Proteinase activity gels were performed according to Michaud, Nguyen-Quoc, Bernier-Vadnais, Faye & Yelle (1994). Samples were equalized by fresh weight (2.5 mg FW), mixed with sample loading buffer (same as for nucleases), and directly loaded onto 10% (w/v) SDS-PAGE gels containing 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) in the resolving gel matrix. After electrophoresis, gels were transferred to a 2.5% (v/v) Triton X-100 solution to facilitate re-naturation of proteinases for 30 min at 25°C. Gels were then immersed in pH 5.0 proteolytic buffer consisting of 50 mM sodium acetate, 5 mM L-cysteine, and 0.1% (v/v) Triton X-100 for 20 h at 37°C. Visualization was accomplished by placing gels on staining solution containing 0.1% (w/v)

Coomassie brilliant blue in 25% (v/v) isopropanol, and 10% (v/v) acetic acid for 2 h at 25°C.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting

Sample proteins were separated by 12% (w/v) SDS-PAGE (Laemmli 1970). Samples were equalized by protein content (20 μg per lane), homogenized in pH 7.3 loading buffer consisting of 50mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.01% (w/v) phenol red, boiled for 2 min, and then centrifuged at 7,000 g for 5 min at 25°C before loading.

Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) using an electrophoretic transfer cell (Model Mini-Trans-Blot, Bio-Rad Laboratories, Hercules, CA) at 100V for 1 h, and probed with polyclonal antibodies, rabbit anti-*Zea mays* leaf MDHAR (Ushimaru, Maki, Sano, Koshiba, Asada & Tsuji 1997) diluted 1:1000 and rabbit anti-spinach leaf GR (Tanaka, Sano, Ishizuka, Kitta & Kawamura 1994) diluted 1:4000. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) was used as secondary antibody diluted 1:2000 with blocking buffer containing 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 0.5% (v/v) Tween-20. Western blots were visualized by chemiluminescence using the ECL Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical Analysis

The experiment was designed and analyzed as a split-split block with three factors: 1) genotypes, 2) duration of exposure to natural sunlight, and 3) presence or absence of UV radiation. Analysis of variance and mean separation were only performed after data met the assumption of normality, which in some cases was achieved by transforming using the ladder of powers ($x=y^p$). When statistical differences were found, Tukey HSD test ($p < 0.05$) was used for mean separation. The analysis was performed using the statistical package SAS Institute Inc. (Cary, NC, USA).

RESULTS

Antioxidant metabolites

Reduced AsA, as well as the redox ratio [(AsA/(AsA+DHA)], decreased significantly as duration of exposure increased (Table 1). The rate at which AsA decreased was greater than the rate at which DHA decreased. This was evident from the increase in DHA/AsA ratio as duration of exposure increased (Table 1). In fact, after 2.5 h there was a proportional increase in DHA concentration (6%) with a reduction in AsA (6%), suggesting an inability of the ascorbate pool to quickly respond to the imposed photooxidative stress (Table 1). In contrast, after 5 h of exposure to solar irradiance not only had AsA declined by 30%, but DHA had decreased by 20% as well, suggesting a partial degradation of the entire ascorbate pool (Table 1). AsA and total ascorbate concentrations and the ratios of AsA/(AsA + DHA) and DHA/AsA decreased linearly with increasing duration of exposure, while the decline in DHA and total ascorbate concentrations were non-linear (Table 1).

The glass filter, which removed 95% of UV radiation, had no effect on ascorbate levels (Table 1). Fruit of both *hp-1* mutants (*hpA* and *hpR*), but especially *hpA*, contained significantly higher concentrations of both AsA and DHA than the other genotypes, but their redox ratios or DHA/AsA ratios did not differ (Table 1). When exposed and covered sections of the fruit were compared after 2.5 h and 5 h of exposure, only after 5 h of exposure did exocarp from the sunlight-exposed section of the fruit have less total ascorbate than the covered section of the fruit (Table 2). Although the differences in AsA and DHA concentrations between exposed and covered sections of the fruit were not statistically significant, both of them declined sufficiently to result in a statistically significant decrease in total ascorbate from the photooxidative stress imposed during exposure to solar irradiance.

Both GSH and total glutathione (GSH+GSSG) concentrations decreased linearly in fruit exocarp as duration of exposure increased (Table 3). Neither the redox ratio (GSH/GSH+GSSG) nor the GSSG/GSH ratio changed as duration of exposure increased (Table 3). Just as for ascorbate, UV radiation did not significantly affect glutathione levels in fruit exocarp (Table 3). There were also no statistical differences in glutathione concentrations or ratios among genotypes (Table 3). Whether a section of fruit was covered or exposed to solar irradiance also did not affect these measures of glutathione (Table 2).

Antioxidant enzymes

When enzyme activities were expressed on a fresh weight basis (FW), different trends were observed as duration of exposure increased (Table 4). SOD activity

increased in the exocarp of immature green fruit, while APX, GR, and CAT decreased. DHAR activity also decreased (P=0.056) as duration of exposure increased (Table 4). Only GR and CAT activities were affected by UV radiation, but in opposite manners. GR activity decreased, whereas CAT activity more than doubled in the exocarp when fruit was exposed to UV radiation (Table 4). Exocarp from *hpA* fruit had significantly higher APX and GR activities than the other genotypes (Table 4). When the covered section of fruit was compared to the section exposed to solar irradiance after 2.5 h and 5.0 h, only APX, GR, and CAT activities expressed on a FW basis responded significantly (Table 5). After 5 h of exposure APX activity was lower in exocarp from the exposed section of the fruit compared to the covered section of the same fruit (Table 5). On the other hand, after 2.5 h GR activity on a FW basis in the exocarp of the sunlight-exposed section was 60% greater than in the covered section, but by 5 h GR activity was 54% lower in the exposed section than the covered section (Table 5). CAT activities were lower in the sunlight-exposed section compared to the covered section after 2.5 h and 5 h of exposure (Table 5).

Exocarp of immature fruit of both *hp-1* mutants had the highest protein concentrations compared to their parents and the other mutants, except *aa* (Table 6). Protein concentrations decreased linearly as duration of exposure increased (Table 6). After 5 h of exposure to solar irradiance there was only half of the initial protein concentration in the exocarp (Table 6). The protein concentration was also lower in exocarp from sections of fruit exposed to sunlight compared to sections of fruit that were covered (Table 5). UV radiation contributed to this decline in protein concentration,

because fruit that were not exposed to UV radiation had 20% higher protein concentrations than fruit that were exposed to UV radiation (Table 6).

Exocarp of *hpR* had the lowest APX activity per unit mass of protein, but similar to AC and *aa* exocarp (Table 6). In contrast, *Del* had much higher CAT specific activity than the other genotypes (Table 6). Even though not significantly different from the other genotypes, the SOD, MDHAR, and GR activities of *Del* appeared much higher, partially due to the lower protein concentration in the exocarp of this mutant. All enzyme activities drastically increased as duration of exposure progressed when expressed on a protein basis, but this was at least partially due to the reduced concentration of total protein (Table 6). These increases in enzyme activities with duration of exposure followed linear trends, except for CAT activity. Only APX and MDHAR specific activities were significantly higher in exocarp from fruit exposed to UV radiation compared to fruit from which UV radiation was excluded (Table 6). For all enzymes, except CAT, specific activities were significantly higher in exocarp from the sunlight-exposed section of fruit than exocarp from the covered section at either 2.5 h or 5 h of exposure (Table 5).

SDS-PAGE immunoblotting confirmed that the expression of MDHAR protein in the exocarp of immature green fruit increased from 0 and 2.5 h to 5 h of sun exposure, while GR protein expression remained stable (Fig. 1). No clear differences were observed between the presence or absence of UV light, or between sun-exposed or covered fruit sections after 2.5 h or 5h in either genotype (data not shown). Similarly, both genotypes, *hpA* and AC, showed similar expression levels of MDHAR and GR across treatments (data not shown).

Lipid, DNA and protein damage

Lipid peroxidation, nuclease activity, and proteinase activity gels were only studied in the most apparently tolerant genotype to photooxidative stress, *hpA*, and its parent AC.

MDA levels were not statistically different in the exocarp of immature green fruit for any of the factors studied: 1) duration of exposure, 2) presence or absence of UV radiation, 3) covered or exposed sections of fruit, or 4) genotypes (data not shown). The mean MDA concentration for all of these factors was 11.1 nmol g^{-1} FW.

Nuclease activity, expressed on a FW or protein basis, did not change as duration of exposure increased (data not shown). There were no statistical differences between genotypes, UV radiation, or fruit surface exposure (data not shown). The mean nuclease activity across all factors was 0.31 ΔOD_{260} mg^{-1} protein and 1.03 ΔOD_{260} g^{-1} FW. The lack of differences among treatments in nuclease activity was confirmed by activity gels (Fig. 1) of both genotypes (data not shown). Proteinase activity gels also showed no differences among treatments (Fig. 1) and genotypes (data not shown).

DISCUSSION

Lipid peroxidation, measured directly as MDA content (a lipid peroxidation end-product) and indirectly as nuclease and proteinase activities, was not significantly different among photooxidative stress treatments or between *hpA* and AC. This indicates that even though AC represented a susceptible genotype to sunscald injury according to preliminary studies, it did not show higher level of lipid, DNA, or protein damage, by the techniques used, than the most visually tolerant genotype tested, *hpA*.

Average MDA content in the samples fell within the normal range reported for non-stressed leaves (Hung & Kao 2004; Liu & Huang 2000).

Indirect DNA damage measured as nuclease activity, which degrade damaged DNA molecules, or protein damage, measured as proteinase activity, did not increase under the imposed photooxidative stress conditions (Fig. 1) in either *hpA* or AC (data not shown). This indicates that the constitutive level of activity of these enzymes might have been enough to cope with the level of damage incurred by the imposed photooxidative stress or that the nuclease and proteinase activities were not sufficiently sensitive to be related to DNA damage and protein oxidation under our experimental conditions. Although, several studies have shown increased nuclease activity in senescing leaf tissue (Langston, Bai & Jones 2005; Lers, Lomaniec, Burd & Khalchitski 2001) and ripening fruit (Lers *et al.* 2001), as well as during other programmed cell death events, such as pathogen attack (Mittler & Lamb 1995). Direct measurement of carbonyl derivatives from oxidized proteins (Levine, Williams, Stadtman & Shacter 1994) might be a more sensitive method for evaluating protein damage during photooxidative stress in fruit.

Our results suggest that antioxidant metabolites and enzymes could have been exerting some degree of protection from photooxidative damage to lipids, DNA, and proteins during our experimental stress conditions. This is, in fact, supported by the significant increase in activities of antioxidant enzymes as duration of exposure increased (Table 6). If the photooxidative stress conditions would have persisted for a longer duration, however, the antioxidant systems would probably have been overcome

at some point, with resulting accumulation of lipid peroxidation products, damaged DNA, and oxidized proteins leading to permanent photooxidative damage.

The constant nuclease and proteinase activities as duration of exposure increased (Fig. 1) either in *hpA* or AC (data not shown) also suggest that these enzymes appear to be more stable at higher temperatures than antioxidant enzymes, whose activities decreased when expressed on a fresh weight basis.

In agreement with Adegoroye & Jollife (1987) and Prohens, Miró, Rodriguez-Burruezo, Chiva, Verdú G. & Nuez (2004), AsA levels decreased in fruit exocarp as duration of exposure increased (Table 1). Similar to our results, a decline in ascorbate content as a response to imposed oxidative stress in leaves has been reported in other studies (Wise & Naylor 1987; Sairam, Deshmukh, & Saxena 1998). On the other hand, plant acclimation and/or plant tolerance to oxidative/photooxidative stress have resulted in higher ascorbate contents in both leaves and fruits (Gatzek, Wheeler & Smirnoff 2002; Logan, Demmig-Adams & Adams III 1998).

Although fruit ripening has been associated with oxidative stress, trends vary in AsA levels in different and even similar species (Andrews *et al.* 2004; Jiménez, Creissen, Kular, Firmin, Robinson, Verhoeyen & Mullineaux 2002; Lentheric, Pinto, Vendrell & Larrigaudiere 1999; Torres 2001). In addition, unlike the effects of UV-B radiation on leaves (Hideg, Mano, Ohno & Asada 1997), there was no effect on the ascorbate pool by the presence or absence of natural UV radiation in our study (Table 1). Our results also suggest that both solar irradiance and high temperatures were equally responsible for the decline in reduced AsA and DHA (Table 2). Similar to the

findings of Foyer, Dujardyn & Lemoine (1989) for leaves, there were no differences in the redox state of ascorbate between covered and sunlight-exposed fruit (Table 2).

As found in this study and also reported by Andrews *et al*. (2004), the *hp*-1 mutation considerably increased the fruit exocarp's ascorbate pool (Table 1). Since ascorbate decreased across genotypes as photooxidative stress progressed, the higher ascorbate concentration in the exocarp of *hp-1* might be one advantage to the antioxidant system that confers this mutant with a greater degree of tolerance to photooxidative stress compared to the other genotypes (see Chapter 2).

Although glutathione has been used as an oxidative stress indicator in plants (Grill, Tausz & Kok 2001), there is conflicting evidence about glutathione responses under oxidative stress conditions. While some studies have found an increase in glutathione synthesis in response to oxidative stress (Kumar & Knowles 1996, Sgherri & Navari 1995), others have found no increase in GSH with environmental stresses (Tausz, Šircelj & Grill 2004). Our results are in agreement with this last group, we did not find an increase in either GSH or GSSG as photooxidative stress progressed (Table 3). This suggests that response mechanisms against oxidative stress are more complex, involving other cellular antioxidant systems besides glutathione. As suggested by Tausz *et al*. (2004), it is also possible that in our study the fruit had only reached an initial state of acclimation to photooxidative stress. If the stress was more sustained, there would be a period of dynamic change in the glutathione pool, and possibly the ascorbate pool as well, giving cells the potential to increase their levels of antioxidant metabolites sufficiently to reach either a protective steady-state, or fail and die. This

response would explain these findings of increased GSH levels in plants submitted to long-term environmental stresses (Polle & Rennenberg 1992)

Our data suggest that increasing GR activity (Table 5) was able to maintain the glutathione redox state (GSH/GSH+GSSG) at over 0.94 throughout exposure of the fruit to photooxidative stress (Table 2), thereby reducing GSSG to GSH via a NADPH-dependent reaction (Carlberg & Mannervik 1985).

The decrease in activities of most of the antioxidant enzymes on a FW basis as the duration of exposure increased (Table 4), indicates that they are more sensitive to high light and temperatures than nucleases and proteases, as shown by activity gels (Fig. 1). However, despite the decline in total protein during exposure to solar radiation, the activities of all antioxidant enzymes on a protein basis dramatically increased as photooxidative stress progressed (Table 6). These results signify that the activities of these enzymes were conserved, perhaps because they were not targeted by proteinases under these environmental conditions imposed in our study. Furthermore, the amount of MDHAR and GR as a fraction of total protein increased or remained the same, respectively, as photooxidative stress progressed (Fig. 2), indicating that these enzymes were conserved under our stress conditions. This may represent an acclimation response to photooxidative stress. Indeed, MDHAR mRNA was found to increase in response to oxidative stresses generated by ozone, hydrogen peroxide, and methyl-viologen (Yoon, Lee, Lee, Kim & Jo 2004). Similarly, increases in mRNA have been observed for other antioxidant enzymes, such as APX (Park, Ryu, Jang, Kwon, Kim & Kwak 2004) and DHAR (Urano, Nakagawa, Maki, Masumura, Tanaka, Murata & Ushimaru 2000), during oxidative stress.

The presence of UV radiation (+UV) increased APX and MDHAR activities on a protein basis (Table 6), and CAT activity on a FW basis (Table 4). Similarly, enhanced peroxidase (including APX) activities in *Arabidopsis thaliana* leaves exposed to UV-B irradiation were previously reported by Rao, Paliyath & Ormrod (1996). It was reported that UV irradiation mainly stimulated the production of $O_2^{\cdot -}$ instead of $^1O_2$ in leaves, whereas mostly $^1O_2$ production was stimulated in leaves exposed to strong visible light (Hideg, Barta, Kalai, Vass, Hideg & Asada 2002).

Drought and chilling stress, as well as other stresses, have been associated with oxidative stress, but for both of these stresses different biochemical responses by antioxidant components have been reported (Gechev, Willekens, Van Montagu, Inze, Van Camp, Toneva & Minkov 2003). In our study, all antioxidant enzyme activities, except CAT, were higher on a protein basis as duration of exposure increased in the exocarp of both the sunlight-exposed and covered sections of fruit (Table 5). This indicates that high temperatures, even without the stress imposed by visible light, produced a similar response in these enzymes of the antioxidant systems in response to an increase in ROS flux.

The *hp-1* mutants, particularly *hpA*, along with having greater ascorbate pools compared to its parent and other mutants (Table 1), also presented significantly higher APX and GR activities (Table 4). The increased APX activity of *hp-1* might be a consequence of greater AsA availability, while the increased GR activity might be due to a higher demand for reduction of oxidized glutathione (GSSH) in order to recycle DHA to AsA.

Finally, our results suggest that during photooxidative stress in chlorophyll-containing fruit exposed to solar irradiance there is an increase in ROS flux, probably due to decreased in intrinsic PSII efficiency (see Chapter 2). When exposed to photooxidative stress, antioxidant metabolites initially decrease and antioxidant enzyme (SOD, APX, DHAR, MDHAR, GR, CAT) activities per unit protein significantly increase in order to reduce ROS and maintain the ascorbate and glutathione pools in their reduced forms, respectively. These mechanisms prevent the accumulation of lipid peroxidation products and probably prevent damage to DNA and oxidization of proteins, at least as long as these antioxidant systems are able to maintain the flux of ROS within functional limits.

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LEGENDS

Figure 1. SDS-PAGE and immunoblots of MDHAR and GR from immature green *hp-A* tomato exocarp samples (20 μg protein lane^{-1}) from photooxidative stress treatments indicated by duration of exposure (0, 2 or 5 h), presence or absence of UV radiation (+UV or −UV), and covered (C) or exposed (E) fruit sections. MDHAR (47 kD) cross-reacted with anti-*Zea mays* MDHAR, and GR (58 kD) cross-reacted with anti-spinach GR.

Figure 2. Effect of photooxidative stress treatments indicated by duration of exposure (0, 2 or 5 h), presence or absence of UV radiation (+UV or −UV) and covered (C) or exposed (E) immature green fruit sections on nuclease (top) and proteinase (bottom) activity gels from *hp-A* mutant. Proteins for nuclease activity were resolved on 10% SDS-PAGE gels containing ssDNA as a substrate, and for proteinase activity on 15% SDA-PAGE gel containing BSA as a substrate. Both gels were loaded with equal fresh sample weight, that is, nucleases 0.25 mg FW and proteinases 2.5 mg FW.

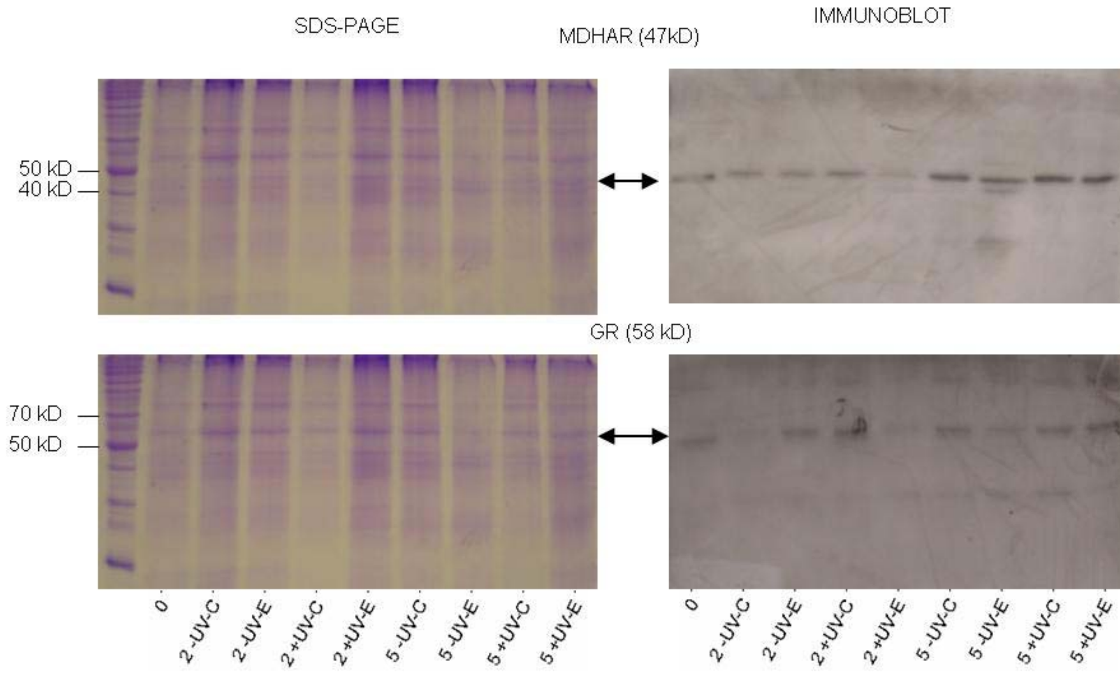

Figure 1

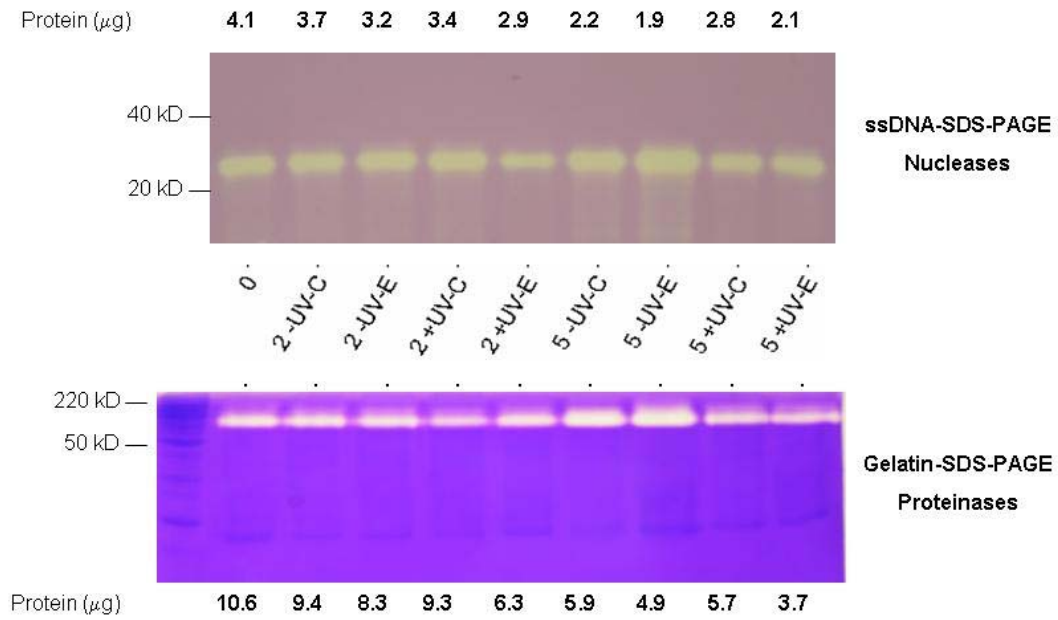

Figure 2

Table 1. Reduced ascorbic acid (AsA), dehydroascorbic acid (DHA), and total AsA+DHA concentrations, and the redox (AsA/AsA+DHA) and DHA/AsA ratios in exocarp of immature green fruit of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultravioltet (+UV or −UV) radiation (C) on exposed sections of immature green fruit. Trend contrasts P values represent a Bonferoni adjustment.

Factors	Reduced AsA (μmol g^{-1} FW)	DHA (μmol g^{-1} FW)	Total AsA (μmol g^{-1} FW)	Redox Red.AsA/Total	DHA/Red. AsA
Genotypes (A)					
'Ailsa Craig'	1.59 b[Z]	2.55 ab	4.14 b	0.36	1.99
Aa	1.80 b	3.06 a	4.86 bc	0.36	1.92
B	1.86 bc	2.90 a	4.76 bc	0.37	1.79
Del	1.64 b	2.85 a	4.49 bc	0.33	2.09
hpA	3.04 d	4.91 c	7.96 d	0.37	1.78
'Rutgers'	1.18 a	1.55 b	2.73 a	0.38	1.54
hpR	2.42 c	3.22 a	5.64 c	0.42	1.71
P value	<.0001	<.0001	<.0001	0.612	0.643
Duration (h) (B)					
0	2.20	3.09	5.29	0.41	1.58
2.5	2.07	3.29	5.36	0.36	1.82
5.0	1.53	2.64	4.17	0.33	2.14
P value Linear	<.0001	0.066	0.001	0.004	<.0001
P value Non-linear	0.140	0.029	0.030	0.451	0.568
(A) x (B) P value	0.545	0.082	0.764	0.643	0.380
UV radiation (C)					
+ UV	1.95	2.96	4.91	0.37	1.81
– UV	1.92	3.05	4.98	0.37	1.86
P value	0.811	0.687	0.755	0.849	0.355
(A) x (C) P value	0.903	0.956	0.755	0.934	0.789
(B) x (C) P value	0.583	0.228	0.481	0.119	0.055
(A)x(B)x(C) P value	0.990	0.881	0.979	0.953	0.481

[Z] Different letters within columns indicate statistical differences. Tukey (p<0.05).

Table 2. Reduced ascorbic acid (AsA), dehydroascorbic acid (DHA), total AsA+DHA concentrations, and redox (AsA/AsA+DHA) and DHA/AsA ratios in exocarp of immature green fruit from exposed and covered sections of immature green fruit. P value indicated for comparison within rows.

Variable	Duration of exposure (h)	Exposed	Covered	P value
Reduced (AsA) (μmol g^{-1} FW)	2.5	2.20	2.37	0.106
	5.0	1.52	1.89	0.057
DHA (μmol g^{-1} FW)	2.5	3.39	3.42	0.983
	5.0	2.60	3.08	0.072
Tot. Ascorbate (μmol g^{-1} FW)	2.5	5.56	5.79	0.401
	5.0	4.12	4.98	0.026
Redox AsA / Tot.	2.5	0.41	0.40	0.959
	5.0	0.34	0.37	0.298
DHA / AsA	2.5	1.74	1.68	0.592
	5.0	2.27	2.08	0.616
GSH (μmol g^{-1} FW)	2.5	0.208	0.189	0.083
	5.0	0.138	0.154	0.091
GSSG (μmol g^{-1} FW)	2.5	0.005	0.004	0.176
	5.0	0.004	0.006	0.214
Tot. (GSH+GSSG) (μmol g^{-1} FW)	2.5	0.213	0.194	0.081
	5.0	0.142	0.160	0.135
GSH / Tot.	2.5	0.980	0.980	0.736
	5.0	0.930	0.990	0.106
GSSG / GSH	2.5	0.024	0.033	0.989
	5.0	0.089	0.069	0.639

Table 3. Reduced (GSH), oxidized (GSSG), and total glutathione (GSH+GSSG) concentrations, and the redox (GSH/GSH+GSSG) and GSSG/GSH ratios in exocarp of immature green fruit of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (+UV and −UV) radiation (C) on exposed sections of immature green fruit. Trend contrasts P values represent a Bonferoni adjustment.

Factors	GSH (nmol g^{-1} FW)	GSSG (nmol g^{-1} FW)	Total (GSH+GSSG) (nmol g^{-1} FW)	Redox GSH/(GSH+GSSG)	GSSG/GSH
Genotypes (A)					
'Ailsa Craig'	179	4.9	184	1.00	0.029
Aa	181	4.4	185	0.99	0.026
B	194	2.2	196	0.99	0.019
Del	197	6.0	203	0.94	0.168
hpA	201	6.2	206	0.98	0.058
'Rutgers'	208	5.1	213	0.94	0.806
hpR	185	5.0	190	0.96	0.113
P value	0.431	0.527	0.412	0.596	0.186
Duration (h) (B)					
0	234	5.1	239	0.98	0.025
2.5	207	5.0	212	0.98	0.025
5.0	138	4.2	142	0.94	0.088
P value Linear	<.0001	0.840	<.0001	0.782	0.082
P value Non-linear	0.140	0.920	0.142	0.654	0.420
(A) x (B) P value	0.429	0.614	0.723	0.975	0.483
UV radiation (C)					
+ UV	184	5.0	188	0.97	0.269
- UV	200	5.1	205	0.97	0.070
P value	0.245	0.354	0.234	0.925	0.139
(A) x (C) P value	0.499	0.859	0.512	0.678	0.730
(B) x (C) P value	0.601	0.707	0.585	0.837	0.429
(A)x(B)x(C) P value	0.772	0.936	0.732	0.654	0.855

Table 4. Antioxidant enzyme (SOD, APX, DHAR, MDHAR, GR, CAT) activities expressed on a fresh weight (FW) basis from exocarp of immature green fruit of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (+UV and −UV) radiation (C) on sections of exposed immature green fruit. Trend contrasts P values represent a Bonferoni adjustment.

Factors	SOD (units min^{-1} g^{-1}FW)	APX (μmol AsA min^{-1} g^{-1} FW)	DHAR (nmol AsA min^{-1} g^{-1} FW)	MDHAR (μmol NADH min^{-1} g^{-1} FW)	GR (μmol NADPH min^{-1} g^{-1} FW)	CAT (AU min^{-1} g^{-1} FW)
Genotypes (A)						
'Ailsa Craig'	178	12.7 ab[Z]	1493	5.8	1.60 b	0.94
Aa	173	12.5 ab	1853	8.7	1.37 ab	0.77
B	183	12.1 ab	2070	9.7	1.41 ab	0.94
Del	170	11.5 ab	1424	7.9	1.37 ab	1.12
hpA	164	18.0 c	2071	6.1	2.10 c	0.58
'Rutgers'	169	11.0 ab	1806	5.1	1.04 a	1.03
hpR	184	11.8 b	2373	6.2	1.67 bc	1.29
P value	0.683	0.005	0.089	0.096	0.020	0.370
Duration (h) (B)						
0	168	13.7	2110	6.7	2.80	1.53
2.5	183	13.9	1804	6.5	1.13	0.65
5.0	172.0	10.9	1724	8.2	0.61	0.66
P value Linear	1.120	0.003	0.056	0.209	<.0001	<.0001
P value Non-linear	0.017	0.082	0.950	0.300	0.001	0.006
(A) x (B) P value	0.308	0.518	0.683	0.078	0.583	0.695
UV radiation (C)						
+ UV	176.3	12.1	1740	7.5	0.78	0.932
- UV	179.8	12.4	1683	7.4	1.00	0.409
P value	0.635	0.471	0.436	0.117	0.047	0.026
(A) x (C) P value	0.114	0.319	0.729	0.743	0.470	0.085
(B) x (C) P value	0.876	0.478	0.656	0.293	0.588	0.888
(A)x(B)x(C) P value	0.365	0.332	0.587	0.880	0.861	0.598

[Z] Different letters within columns indicate statistical differences. Tukey ($p < 0.05$).

Table 5. Antioxidant enzyme (SOD, APX, DHAR, MDHAR, GR, CAT) activities expressed on fresh weight (FW) and protein bases, and total protein in exocarp from exposed and covered sections of immature green fruit. P value indicated for comparison within rows.

Variable	Duration of exposure (h)	Exposed	Covered	P value
SOD (units $min^{-1} g^{-1}$ FW)	2.5	157	183	0.094
	5.0	173	162	0.444
APX (μmol AsA $min^{-1} g^{-1}$ FW)	2.5	13.3	13.9	0.083
	5.0	10.8	12.9	0.000
DHAR (nmol AsA $min^{-1} g^{-1}$ FW)	2.5	1467	1803	0.559
	5.0	1707	1572	0.287
MDHAR (μmol NADH $min^{-1} g^{-1}$ FW)	2.5	4.5	6.6	0.509
	5.0	8.3	6.3	0.097
GR (μmol NADPH $min^{-1} g^{-1}$ FW)	2.5	1.78	1.11	0.001
	5.0	0.63	1.36	<.0001
CAT (AU $min^{-1} g^{-1}$ FW)	2.5	0.68	1.23	0.004
	5.0	0.66	1.11	0.031
PROT (mg g^{-1} FW)	2.5	2.9	3.3	0.006
	5.0	1.8	2.7	0.003
SOD (units $min^{-1} g^{-1}$ prot.)	2.5	87.2	73.8	0.003
	5.0	711	186	0.001
APX (μmol AsA $min^{-1} g^{-1}$ prot.)	2.5	6.1	5.3	0.128
	5.0	30.3	10.6	0.002
DHAR (nmol AsA $min^{-1} g^{-1}$ prot.)	2.5	942	684	0.087
	5.0	1459	897	0.001
MDHAR (μmol NADH $min^{-1} g^{-1}$ prot.)	2.5	337	243	0.293
	5.0	3199	553	0.002
GR (μmol NADPH $min^{-1} g^{-1}$ prot.)	2.5	44.2	64.8	0.001
	5.0	97.3	65.7	0.236
CAT (AU $min^{-1} g^{-1}$ prot.)	2.5	0.08	0.11	0.088
	5.0	2.37	0.20	0.445

Table 6. Total protein and antioxidant enzymes (SOD, APX, DHAR, MDHAR, GR, CAT) activities expressed in protein basis in exocarp of immature green fruit of different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (+UV and –UV) radiation (C) on sections of exposed immature green fruit. Trend contrasts P values represent a Bonferoni adjustment.

Factors	PROT (mg g^{-1} FW)	SOD (units min^{-1} g^{-1} prot.)	APX (μmol AsA min^{-1} g^{-1} prot.)	DHAR (nmol AsA min^{-1} g^{-1} prot.)	MDHAR (nmol NADH min^{-1} g^{-1} prot.)	GR (nmol NADPH min^{-1} g^{-1} prot.)	CAT (AU min^{-1} g^{-1} prot.)
Genotypes (A)							
'Ailsa Craig'	3.01 a[Z]	104	6.41 ab	872	462	50.8	0.13 a
Aa	3.10 abc	90.3	5.08 ab	809	533	45.3	0.06 a
B	2.36 a	115	6.22 a	1099	466	58.4	0.12 a
Del	1.91 a	1646	10.30 a	1328	4188	191.3	4.72 c
hpA	3.73 c	359	5.13 ab	689	1759	65.9	0.03 a
'Rutgers'	2.45 a	348	9.33 a	1323	1087	66.9	0.87 b
hpR	3.24 bc	1045	4.11 b	888	271	50.9	0.20 a
P value	<.0001	0.139	0.014	0.303	0.120	0.406	0.049
Duration (h) (B)							
0	3.74	56.9	4.02	623	202	84.1	0.11
2.5	2.89	87.2	6.06	941	333	44.4	0.08
5.0	1.91	1064	29.6	1438	3113	94.5	2.34
P value Linear	<.0001	<.0001	<.0001	0.001	<.0001	0.002	0.284
P value Non-linear	0.950	0.126	0.322	0.948	0.070	0.220	<.0001
(A) x (B) P value	0.138	0.304	0.389	0.247	0.453	0.355	0.253
UV radiation (C)							
+ UV	2.18	676	19.4	1289	2616	92.4	1.30
- UV	2.62	473	8.4	1014	967	48.5	1.26
P value	0.022	0.292	0.009	0.474	0.026	0.426	0.368
(A) x (C) P value	0.415	0.525	0.271	0.527	0.839	0.115	0.183
(B) x (C) P value	0.926	0.092	0.130	0.217	0.067	0.075	0.604
(A) x (B) x (C) P value	0.055	0.426	0.372	0.242	0.263	0.617	0.414

[Z] Different letters within columns indicate statistical differences. Tukey (p<0.05)

CHAPTER 4

DISPOSITION OF SELECTED FLAVONOIDS IN FRUIT TISSUES OF VARIOUS TOMATO GENOTYPES

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ABSTRACT

Flavonoids, an important group of secondary metabolites present in fruits and vegetables, are the subject of extensive research because of their potential health benefits. In the present study, we used enzymatic hydrolysis of the glycosylated flavonols, quercetin and kaempferol, and the flavanone naringin, to obtain their sugar-free aglycones. In addition, we employed a validated HPLC method to obtain the chiral disposition of the aglycone naringenin enantiomers. These analyses were conducted on different fruit tissues (exocarp, mesocarp, and seed cavity) of field-grown tomato (Lycopersicon esculentum Mill.) mutants (*anthocyanin abscent, atroviolacea,* and *high pigment-1*) and their nearly isogenic parent ('Ailsa Craig') at three maturity stages (immature green, "breaker", and red ripe). Significantly higher levels of all flavonoids were obtained with enzymatic hydrolysis than those previously reported using acid hydrolysis. Presumably, this was due to more complete hydrolysis and release of aglycones by the β-glucuronidase enzyme. Glycosylated S-naringin was the predominant enantiomer in all fruit tissues, although free R- and S-naringenin were detected in both exocarp and mesocarp tissues. While there was significantly more quercetin than kaempferol in exocarp tissue, they were present in about equal concentrations in the mesocarp. Quercetin concentrations were higher in the exocarp and mesocarp of immature green and "breaker" fruit of the *high pigment-1* mutant than in the other genotypes, supporting the observed photo protection and potential health benefits of the *high pigment-1* tomato genotype.

KEYWORDS

Quercetin, kaempferol, naringenin, enantiomer, maturity stage, HPLC, enzymatic hydrolysis

INTRODUCTION

Flavonoids are an important group of secondary metabolites in plants derived from the phenylpropanoid biochemical pathway. Their basic structure consists of two aromatic benzene rings separated by an oxygenated heterocyclic ring. Although they are present in many plant tissues, they are usually located only in cell vacuoles. The flavonoids have important functions in plants, including defense against pathogens and protection against ultraviolet B (UV-B) radiation (*1, 2, 3*). In mammals, they have been shown to have multiple health benefits, as antioxidants and also act as anti-inflammatory, anti-cancer, and anti-viral compounds (*4, 5, 6, 7*). Moreover, flavonoids also influence sensory characteristics, such as, the flavanones that are responsible for the bitter flavor of citrus fruits (*8*).

Flavonols and flavanones are the major subclasses of flavonoids in fruits. These flavonoids are usually conjugated with sugar molecules, such as glucose, rhamnose, rutinose, galactose, and arabinose (*O*-glycosides), which increase their polarity so that they can be stored in vacuoles. However, flavonols and flavanones can also be present without an attached sugar molecule, in which case they are referred to as aglycones. The two major flavonols in fruits are quercetin and kaempferol, and in tomato they are primarily conjugated with a sugar molecule (*9, 10*). Le Gall *et al.* (*11)* identified quercetin-3-O-rutinoside (rutin), a type of kaempferol glycoside, and naringenin-7-O-glucoside and naringenin chalcone as major flavonoid components of whole ripe tomato fruits. Naringenin is a flavanone synthesized upstream from quercetin and kaempferol (**Scheme 1, section 2**). Different ratios of the chiral flavanone 7-O-glycoside, naringin [(+/-) 4',5,7-trihydroxyflavanone 7-rhamnoglucoside], to its aglycone, naringenin [(+/-)

4',5,7-trihydroxyflavanone], are found in tomato and citrus fruits (*12, 13, 14*). Compared to citrus fruits, tomatoes have relatively more of the aglycone naringenin than naringin. Moreover, several studies have not been able to find neither naringenin-7-O-rhamnoglucoside nor naringenin-7-O-glucoside in tomato paste (*15, 16, 17, 18*).

Although flavonoids are abundant in tomato fruit, the purported anti-oxidant properties of tomato consumption have been mostly attributed to the fruit's major red carotenoid pigment, lycopene (*7*). However, when lycopene was administered along with polyphenols, including flavonoids, lycopene's antioxidant properties were enhanced (*19*).

In the present study we investigate naringenin, together with quercetin and kaempferol profiles in tomato fruit tissues (exocarp, mesocarp, and seed cavity) at different fruit maturity stages of four tomato genotypes. Because naringenin is a chiral compound, either one or both of its enantiomers may be the active component(s) with regard to potential health benefits. This study also presents, for the first time, the quantitative analysis of naringenin and naringin enantiomers in tomatoes using a validated stereo-selective, isocratic, reversed-phase high performance liquid chromatography (HPLC) method. The quantification of all flavonoids involved the use of enzymatic hydrolysis for the removal of the sugar moiety instead of the normal acid hydrolysis approach used in similar studies on fruits and vegetables.

MATERIALS AND METHODS

Plant Material. Fruit from four tomato (*Lycopersicon esculentum* Mill.) genotypes, whose seeds were obtained from the C.M. Rick Tomato Genetics Resource Center

(University of California, Davis, USA) were used. The genotypes were: 1) *anthocyanin absent (aa),* whose mutation has not been previously described, 2) *atroviolacea (atv)*, which shows an enhanced photo responsiveness (*20*), 3) *high pigment-1 (hp-1*), a highly studied genotype with a mutation of a negative regulator of the phytochrome signal translator resulting in an exaggerated photo response (*20*), and 4) the nearly-isogenic parent ('Alisa Craig') of these mutants. Some reported traits for these mutants are given in **Table 1**.

Seeds were germinated and seedlings were grown in cell packs in a greenhouse until they had several true leaves, at which time they were transplanted into replicated plots in the field in a randomized complete block design consisting of 4 blocks with six plants per plot. Plants were spaced 1.2 m apart in the rows, with rows 2.4 m apart.

Field Site. The research site was located in Lewiston, Idaho (46°23' N; 116°59' W) at an elevation of 430 m above sea level. The site was level of a uniform Nez Perce silty, clay-loam textured soil (fine, montmorillonitic, mesic Xeric Argialbolls). This region would be classified as desert steppe with summer (June-Sept.) mean maximum temperatures of 30°C on generally cloudless days, with most precipitation occurring only in the winter months.

All plants were irrigated with buried drip tape, one hour per day during the first 30 days after transplanting, and two hours per day from 30 days after planting until the end of the growing season. The output of the emitters was 1.9 L h^{-1}. Plants were surface fertilized with calcium nitrate (30 g per plant) only once during the season, two weeks after transplanting.

Fruit Sampling. Healthy, equal-sized fruits were harvested from each block and genotype at three maturity stages. These stages were: 1) Green immature, 2) Breaker (intermediate), and 4) Red (fully ripen) (Plate 1).

From each fruit, the exocarp (peel, approximately 2 mm thick), mesocarp (flesh), and jelly-like seed cavity (including columella, placenta tissue, and seeds) tissues were separately collected, immediately frozen using liquid N_2, and stored at -80°C. The frozen tissue was later ground to a fine powder using a mortar and pestle and liquid N_2, and stored at -80°C until analysis.

Chemicals and Reagents. HPLC grade methanol, hexane, acetonitrile, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was purchase from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Quercetin, kaempferol, naringenin, 7-ethoxycoumarin, daidzein, and *Helix pomatia* Type-HP-2 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Flavonoid Extraction. Flavonoids were extracted from fresh tissue using a ratio of tissue:100% methanol:hexane of 0.1:1:0.5 (w/v/v). First, powdered tissue was ground with methanol using an homogenizer (Talboys Engineering Corp., Montrose, PA) for 2 min. The slurry was then **transferred** to Eppendorf tubes and hexane was added. After samples were centrifuged at 13,000 g for 10 min, the hexane layer was discarded and the methanol fraction was dried completely under a stream of purified N_2 gas. Samples were maintained at 2°C throughout the extraction procedure.

Dried samples were then re-constituted in mobile phase (400μl) for measurement of naringenin by HPLC, or enzymatically hydrolyzed to measure the total individual flavanones, quercetin, kaempferol, and naringenin.

Enzymatic Hydrolysis. The enzymatic hydrolysis of flavonoids was performed according to the method described by Yañez and Davies (*21*) with some modifications (example for naringenin is shown in Scheme 1, section A). Dried samples from the flavonoid extraction were re-suspended in 0.78 M acetate buffer (pH 4.8), 0.1 M ascorbic acid, and *Helix pomatia* Type-HP-2 β-glucuronidase (100 μl). The mixture was incubated for 17-24 h at 37°C after which samples were centrifuged at 7,000 g for 10 min at room temperature (25°C). Each sample was divided in two equal aliquots, one for naringenin, to which 25 μl of daidzein (1 mg ml^{-1}) was added as internal standard, and the other one for quercetin and kaempferol detections, to which 25 μl of 7-ethoxycoumarin (1 mg ml^{-1}) was added as internal standard. In both aliquots, 1 ml of 100% cold acetonitrile was added and vortexed for 1 min. Samples were then centrifuged at 7,000 g for 5 min at room temperature. Supernatants were transferred to new Eppendorf tubes and dried completely under a stream of purified N_2 gas. Dried samples were then reconstituted in 400 μl mobile phase for naringenin or quercetin and kaempferol assays, vortexed for 1 min and centrifuged at 7,000 g for 5 min. Supernatants were transferred to HPLC vials. In all cases the injection volume was 150 μl.

Standards Solutions. All standards were made at 1 mg ml^{-1} stock solutions in methanol. They were protected from light and stored at –20°C for up to 3 months.

Chromatographic Conditions. Extracts and standards were injected into a Shimadzu HPLC system (Kyoto, Japan), consisting of a LC-10AT VP pump, a SIL-10AF auto injector, a SPD-M10A VP spectrophotometric diode array detector, and a SCL-10A system controller. Integration and collection of data was carried out using the Shimadzu EZ Start 7.1.1. SP1 software (Kyoto, Japan).

Naringenin enantiomers were separated by a Chiralcel OD-RH column (150 mm x 4.5 mm I.D., 5-mm particle size. Chiral Technologies Inc. Exton, PA, USA) under isocratic conditions at 25°C. Separation was carried out using a mobile phase of acetonitrile:water:phosphoric acid (30:70:0.04, v/v/v) and a flow rate of 0.4 ml min^{-1}. Naringenin enantiomers were detected at 292 nm. This stereo-selective, reverse-phase HPLC method has been previously validated and described in detail by Yañez and Davies (2005).

Quercetin and kaempferol were also separated isocratically by a Chiralcel AD-RH column (150 mm x 4.5 mm I.D., 5-mm particle size. Chiral Technologies Inc. Exton, PA). The mobile phase was acetonitrile:water:phosphoric acid (42:58:0.01, v/v/v) and a flow rate of 0.6 ml min^{-1}. Both flavonoids were detected at 370 nm. Mobile phase solvents were filtered and degassed before use.

Statistical Analysis. Flavonoid contents were quantified based on standard curves constructed using peak area ratio (PAR) against standard concentrations. PAR

was obtained by dividing peak area of the compound and peak area of the internal standard. Least squares linear regression was used for this purpose.

The experimental design was a split-block with two factors: A) genotypes, and B) maturity stage. The analysis was carried out separately for three different fruit tissues: 1) exocarp, 2) mesocarp, and 3) seed cavity. Analysis of variance and mean separation were only performed after data met the assumption of normality, which in some cases was achieved by transforming data using the ladder of powers ($x=y^p$). When statistical differences were found, a protected LSD test ($p < 0.05$) was used for mean separation. The analysis was performed using the statistical package SAS Institute Inc. (Cary, NC, USA).

RESULTS

All compounds, quercetin, kaempferol, naringenin enantiomers and internal standards, were separated successfully, without interfering peaks co-eluting with them (**Figure 1A, 1B**). Interestingly, enzymatic hydrolysis to obtain free plus conjugated flavonoid compounds resulted in significantly greater amounts of all compounds than those reported for the commonly used acid hydrolysis method (*22, 23, 24, 25, 15, 26, 9*).

Naringenin: R-, S- naringenin, and their internal standard (IS) eluted at approximately 46, 50, and 25 min, respectively (**Figure 1A**). In all tomato fruit tissues (exocarp, mesocarp, and seed cavity) total amounts of S-naringenin were greater than those of R-naringenin (**Table 2**). In agreement with many other studies (*9*) that indicate

that tomato skin has the highest concentration of flavonoids, the exocarp of tomato fruit contained approximately 92% of total naringenin (R + S configurations). Fruit mesocarp (flesh) and seed cavity contained 4.5% and 3.5%, respectively. When enantiomers were analyzed separately, the percentage of R configuration in the exocarp dropped to 80%, while that in flesh and seed cavity increased to 10% of each tissue type.

Genotype and maturity stage differences varied with the tissue involved. There was a significant interaction between both factors (genotype x maturity stages) in fruit exocarp (**Table 2**). Total naringenin, as well as the R and S configurations, were consistently higher in the exocarp of *hp-1* at the green immature stage (**Figure 2**). Although not always statistically significant, all tomato mutants generally showed higher total naringenin than their parent 'Ailsa Craig' (**Figure 2**). In both mesocarp (flesh) and seed cavity tissues, immature green fruit had the lowest total and R- and S-naringenin concentrations (**Table 1**). In general, there was a prominent increase in both naringenin enantiomers at the breaker stage, which later decreased at the red-ripe stage.

Free naringenin (before enzymatic hydrolysis) accounted for approximately 30% of total, enzymatically hydrolyzed naringenin of fruit exocarp tissue, with most as free S-enantiomer. Therefore, conjugated naringenin, as naringin, would account for about 70% of total naringenin in exocarp tissue.

In all tissue types, concentrations of both R- and S- enantiomers of free naringenin showed significant interactions between genotypes and maturity stages (**Table 3**). In fruit exocarp and mesocarp, genotypes were only statistically different at the breaker maturity stage (**Figures 3 and 4**). In the exocarp, 'Ailsa Craig' showed significantly higher concentrations of R- and S-naringenin than *hp-1* and *atv* fruit

(**Figure 3**), while in the mesocarp *hp-1* fruit showed the greatest concentrations of naringenin enantiomers (**Figure 4**). In contrast, seed cavity tissue from green fruit of 'Ailsa Craig' showed the highest contents of R- and S-naringenin (**Figure 5**).

Quercetin and Kaempferol: Retention times for quercetin, kaempferol, and their internal standard (IS) were approximately 10, 16, and 27 min, respectively (**Figure 1B**).

In fruit exocarp there was an interaction between genotype and maturity stages for both quercetin and kaempferol concentrations (**Table 4**). For both compounds *hp-1* fruit had the highest concentration among genotypes. At the breaker stage, *hp-1* also had the highest total quercetin concentration (**Figure 6**). Only in mesocarp tissue was there an interaction between genotype and maturity stage for quercetin concentration (**Table 3**). Immature green and breaker fruit from *hp-1* showed a significantly higher concentration of quercetin than the other genotypes (**Figure 7**).

Overall, kaempferol accounted for only about 10% of the quercetin in fruit exocarp tissue, but 75% and 110% of the quercetin in fruit mesocarp and in seed cavity tissue, respectively. These differences are also reflected in the quercetin/kaempferol (Q:K) ratio, which was over 7.5 in fruit exocarp, but about 1.0 in mesocarp and seed cavity tissues (**Table 4**). The Q:K ratio differed among genotypes and maturity stages. In fruit exocarp, *hp-1* was almost twice the Q:K ratio compared to the rest of the genotypes, which is a consequence of its elevated quercetin concentration (**Figure 8**). A high Q:K ratio was also apparent, but less pronounced, in fruit mesocarp. Only in fruit exocarp was there an increase in the Q:K ratio as fruit maturity progressed (**Table 4**).

DISCUSSION

In the present work we report for the first time the naringenin enantiomer concentrations in different tomato fruit tissues during development, using a stereospecific HPLC method validated for biological fluids by Yañez and Davies (*21*). The rapid, sensitive and stereo-specific detection of the selected flavonoids obtained with the HPLC methods used were enhanced by enzymatic hydrolysis to detect total aglycones. Furthermore, enzymatic hydrolysis resulted in greater apparent flavonoid detection than reported for acid hydrolysis, which is commonly used for flavonoid analysis of fruits and vegetables (*27, 22, 28, 24, 26, 29, 9*). For example, the level of flavonols (quercetin and kaempferol) in tomato skin reported by Stewart et al. (*9*) was 4 times lower than what we report in the present study. Similarly, the quercetin and kaempferol values reported by Martinez-Valverde et al. (*26*) for whole tomato fruit extracts were about 3 times lower than the present reported values for fruit mesocarp (flesh). Justesen et al. (*24*) reported quercetin concentrations in whole tomato fruit that were almost 5 times lower than those found in fruit mesocarp in the present investigation. One explanation for this is that β-glucuronidase is more specific and efficient in de-glycosylation, and thus capable of freeing a larger number of aglycones (flavonoids) from their sugar molecules, than acid hydrolysis and/or elevated temperatures. This critical hydrolysis step is key to determining the total amount of flavonoids, both glycosylated and aglycones, for quantifying specific flavonoid content of fruits and vegetables by HPLC.

In contrast to Muir et al. (*29*), who did not detect any naringenin aglycones in tomato skin, we found that 30% of total naringenin was present as aglycone in fruit

exocarp. Moreover, we were also able to detect naringenin aglycones in fruit flesh. In contrast, Paganga et al. (*15*) and Wardale (*18*) reported that only the aglycone naringenin was present in tomato fruit.

As in citrus juice (*21*), glycosylated S-naringin was found to be the predominant enantiomer in all tissue types. S-naringenin accounted for 85% of total naringenin in fruit exocarp, and approximately 60% in fruit mesocarp and seed cavity. This information could be important for bioavailability studies, since chirality may have a significant influence on physiology and pharmacological action and disposition (*30*). In the case of naringenin, preliminary studies have shown that S-naringenin has a longer biological half-life than R-naringenin (Personal communication, Dr. Neal Davies, Washington State University).

Quercetin and naringenin have been reported to be the major flavonoids in tomato fruit (*11, 24, 14, 15*). In our study we found that this was true only for fruit exocarp (peel), which had approximately 50% naringenin, 45% quercetin, and 5% kaempferol. In mesocarp (flesh) and seed cavity, however, naringenin, quercetin, and kaempferol were present in approximately equal amounts.

Kaempferol is often not detected in samples of whole tomato fruit (*15, 26*), yet we were able to detect it even in both mesocarp and seed cavity tissues. As for naringenin and quercetin, exocarp contained the highest amount of kaempferol, twice that in flesh and seed cavity tissues. This concentration of kaempferol in the exocarp was at least 7 times higher than that in red-ripe tomato skin reported by Steward *et al.* (9).

Interestingly, the kaempferol concentration gradient between the fruit exocarp and mesocarp did not appear to decline as drastically as did quercetin between these

two tissues. While kaempferol decreased by 53% between the exocarp and mesocarp, quercetin decreased by 93%. Since the accumulation of quercetin glycosides in fruit exocarp plays an important role in UV-B protection (*32*), the plant avoids expenditure of additional energy by not synthesizing excess quercetin in unexposed mesocarp tissue.

In our study there was not a common trend for quercetin, kaempferol, and naringenin concentrations with fruit maturity among genotypes. In agreement with Muir *et al*. (*29*), quercetin levels in fruit exocarp (peel) increased with maturity, although this was not true for the *hp-1* mutant. In contrast, naringenin enantiomers were highest at the breaker stage, declining in red-ripe fruit, a pattern similar to that reported by Muir et al. (*29*) and Le Gall et al. (*11*) for naringenin chalcone, a precursor of naringenin in the biosynthetic pathway.

Differences in flavonoid concentrations among tomato genotypes have been reported in the literature (*26, 14, 9*). Therefore, we also expected to find genotype differences in flavonoid concentrations. All mutants had either similar or higher concentrations of total flavonoids than their parent, 'Ailsa Craig'. Since *aa* apparently does not synthesize anthocyanin in any plant part (*33*), its mutation is likely located at the last step of anthocyanin biosynthesis, that is, downstream from naringenin and dihydrokaempferol precursors. In contrast, the *atv* mutant, described as having enhanced anthocyanins in all plant parts (*33*), did not have higher flavonoid concentrations than the other genotypes tested. On the other hand, the *hp-1* mutant, which has been widely studied due to its increased photo-responsiveness and fruit with higher concentrations of chlorophyll, carotenoids, phenolics, and anthocyanins (*34, 35, 36, 14, 37*), had significantly higher flavonoid concentrations, especially quercetin, than

the other genotypes in this study. Furthermore, the higher flavonoid concentrations of *hp-1* would partially explain its increased tolerance to photo-oxidative stress under natural environmental conditions (*38*). Moreover, Stewart *et al.* (*9*) analyzed a number of genotypes of different fruit colors (red, purple, yellow) and concluded that flavonol content (quercetin and kaempferol) was not necessarily correlated with accumulation of anthocyanins in these genotypes.

It is also important to mention that there were no statistical differences among leaf samples of the tomato genotypes for quercetin, kaempferol, or naringenin concentrations. Their concentrations were on average 1360 μg g^{-1}FW (± 231.2 SD) for quercetin, 97.4 μg g^{-1}FW (± 10.1 SD) for R-naringenin, 95.3 μg g^{-1}FW (± 13.1 SD) for S-naringenin, and 330 μg g^{-1}FW (± 19.1 SD) for kaempferol.

In summary, enzymatic hydrolysis dramatically increased the apparent total flavonoid quantification of tomato fruit tissues. This is the first reported detection of naringenin and naringin enantiomers in tomato tissues. In the future, additional research needs to be done to elucidate the physiological and pharmacological significance of naringenin enantiomers, and the bioavailability and stereospecificity of their actions. Finally, the *hp-1* gene was once again found to be an interesting element to be incorporated into breeding programs for enhancing tomato fruit flavonoid concentrations.

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LEGENDS

Plate 1. Example of tomato fruit maturity stages in *hp-1* mutant.

Scheme 1. Enzymatic hydrolysis converting naringin to naringenin **(A)**; Naringenin flavanone as common precursor of quercetin and kaempferol **(B)**

Figure 1. Typical chromatograms of a fruit tissue extract after enzymatic hydrolysis using β-glucuronidase from *Helix pomatia* type H-2. Chromatogram **A** shows peaks for R-naringenin, S-naringenin, and diadzein (IS). Chromatogram **B** shows quercetin, kaempferol, and 7-ethoxycoumarin peaks.

Figure 2. Genotype x maturity stage interaction for total R-, S-, and total naringenin concentrations in fruit exocarp. Mean separation by protected LSD ($P<0.05$). Mean separation by protected LSD ($P<0.05$). Different letters between genotypes at each maturity stage indicates statistical differences. n.s. indicate non-statistically different.

Figure 3. Genotype x maturity stage interaction for total free (non-conjugated) R-, S-, and total naringenin concentrations in fruit exocarp. Mean separation by protected LSD ($P<0.05$). Different letters between genotypes at each maturity stage indicate statistical differences. n.s. indicate non-statistically different.

Figure 4. Genotype x maturity stage interaction for total free (non-conjugated) R-, S-, and total naringenin concentrations in fruit mesocarp. Mean separation by protected LSD ($P<0.05$). Different letters between genotypes at each maturity stage indicate statistical differences. n.s. indicate non-statistically different.

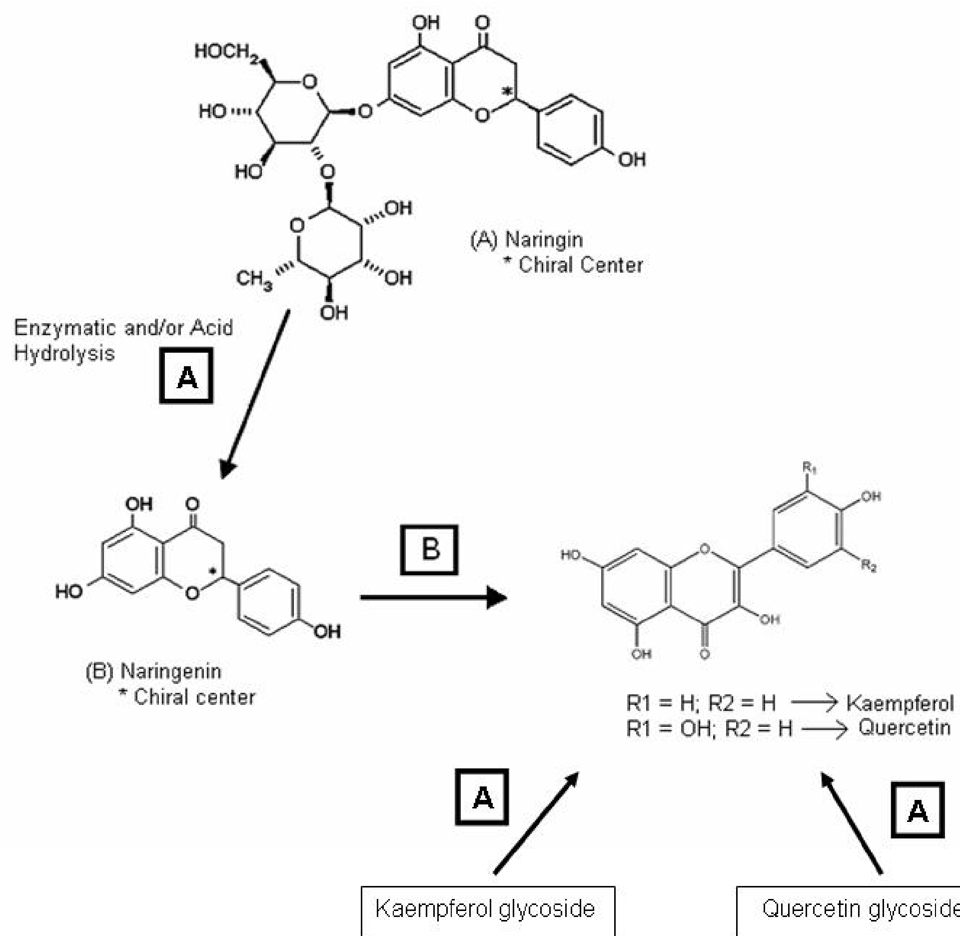
Figure 5. Genotype x maturity stage interaction for total free (non-conjugated) R-, S-, and total naringenin concentrations in fruit seed cavity. Mean separation by protected LSD ($P<0.05$). Different letters between genotypes at each maturity stage indicate statistical differences. n.s. indicate non-statistically different.

Figure 6. Genotype x maturity stage interaction for total kaempferol and quercetin concentrations in fruit exocarp (peel). Mean separation by protected LSD ($P<0.05$). Different letters between genotypes at each maturity stage indicate statistical differences. n.s. indicate non-statistically different.

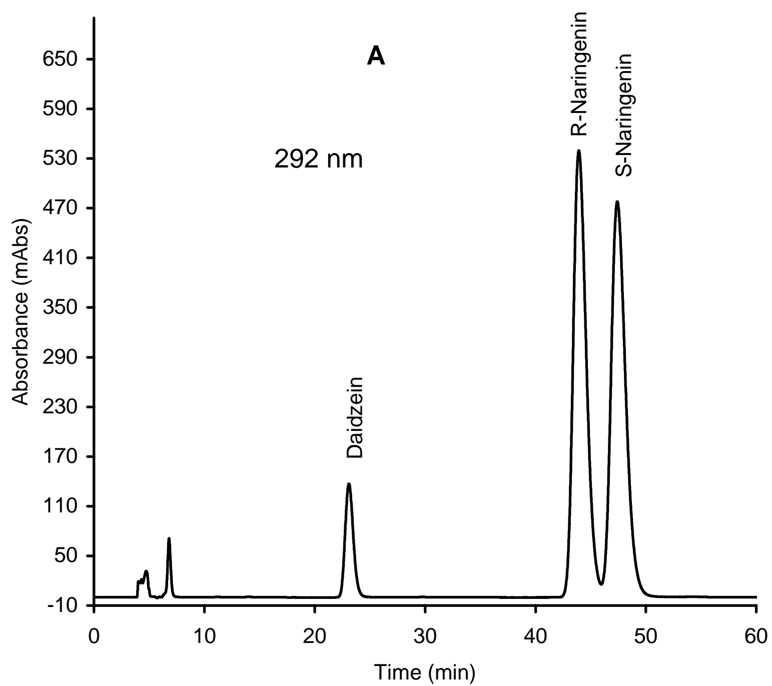
Figure 7. Genotype x maturity stage interaction for total quercetin concentration in fruit mesocarp (flesh). Mean separation by protected LSD ($P<0.05$). Different letters between genotypes at each maturity stage indicate statistical differences. n.s. indicate non-statistically different.

Plate 1

Red -ripe	Breaker-Intermediate	Green - Immature



Scheme 1



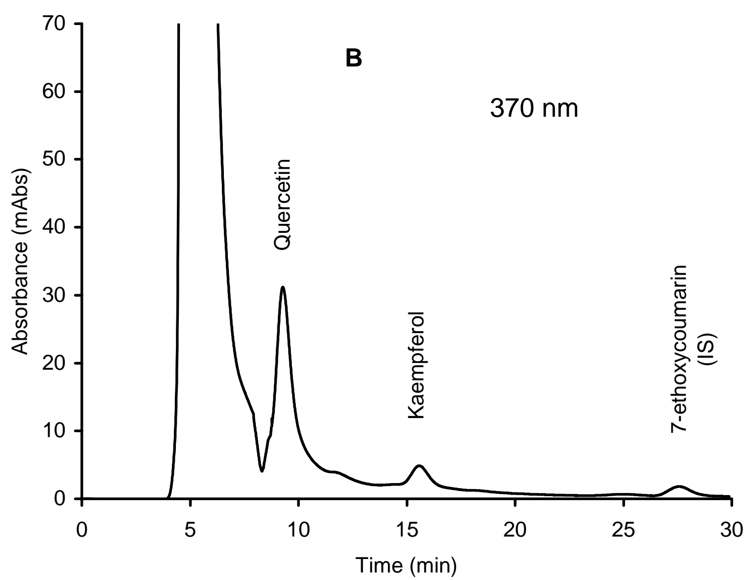


Figure 1

Table 1. Summary of reported traits of tomato genotypes used in this study.

Mutants	Description
anthocyanin absent (*aa*) (LA 3617)	*L. esculentum* Mill., nearly-isogenic in 'Ailsa Craig'. Mutation on chromosome 2. Anthocyanin completely absent in all plant parts (*33, 39*)
atroviolacium (*atv*), LA 3736	*L. esculentum* Mill., nearly-isogenic in 'Ailsa Craig'. Elevated anthocyanin levels in all plant parts. The main anthocyanin present in leaves and stems was identified as peonidin-3-(*p*-coumaryl rutinoside)-5-glucoside (*33, 39*).
high pigment (*hp-1*) (LA 2838A)	*L. esculentum* Mill., nearly-isogenic in 'Ailsa Craig'. Recessive non-allelic mutation in locus 12 (monogenic), first identified in 1917. Chlorophyll, carotenoids (lycopene and β-carotene), and ascorbic acid content of fruit intensified (*35, 36, 37*), also anthocyanins (*20*). This characteristic has also been found in breeding lines with *hp-1* (*36*).

Table 2. Total R-, S-, and total naringenin in fruit exocarp (peel), mesocarp (flesh), and seed cavity in different genotypes (A), fruit maturity stages (B), and their interaction [(A) x (B)].

Factors	Total R-Naringenin (μg g^{-1}FW)			Total S-Naringenin (μg g^{-1}FW)			Total Naringenin (R+S) (μg g^{-1}FW)		
	Exocarp	Mesocarp	Seed Cavity	Exocarp	Mesocarp	Seed Cavity	Exocarp	Mesocarp	Seed Cavity
Genotypes (A)									
'Ailsa Craig'	141	24.0	22.0	910	35.4	27.8	1051	59.4	49.8
aa	191	23.8	22.7	1233	35.4	33.1	1424	59.2	55.8
atv	182	24.5	23.2	985	37.0	32.8	1167	61.4	56.0
hp-1	210	24.3	21.9	1160	42.1	28.4	1370	66.5	50.3
P value	0.002	0.872	0.342	0.002	0.525	0.263	0.001	0.494	0.195
Maturity Stage (B)									
Green	29.6 a	20.2 a[z]	20.8 a	65.0	23.2 a	26.2 a	94.6	43.4 a	47.0 a
Breaker	268 c	22.8 b	22.9 b	2295	47.3 b	34.2 b	2569	70.1 b	57.1 b
Red	248 b	29.5 c	23.6 b	862	41.9 b	31.3 b	1110	71.3 b	54.9 b
P value	<.0001	<.0001	0.001	<.0001	<.0001	0.010	<.0001	<.0001	0.005
(A) x (B) P value	0.007	0.938	0.790	<.0001	0.763	0.936	<.0001	0.872	0.910

[z] Different letters indicate statistical differences. Mean separation by protected LSD (< 0.05)

Table 3. Total R-, S-, and total free naringenin in fruit exocarp (peel), mesocarp (flesh), and seed cavity in different genotypes (A), fruit maturity stages (B), and their interaction [(A) x (B)].

	Total Free R-Naringenin ($\mu g\ g^{-1}FW$)			Total Free S-Naringenin ($\mu g\ g^{-1}FW$)			Total Free Naringenin (R+S) ($\mu g\ g^{-1}FW$)		
Factors	Exocarp	Mesocarp	Seed Cavity	Exocarp	Mesocarp	Seed Cavity	Exocarp	Mesocarp	Seed Cavity
Genotypes (A)									
‘Ailsa Craig’	100	11.5	10.2	360	11.2	10.3	460	22.8	20.5
aa	207	10.4	9.5	212	10.0	9.3	419	20.4	18.8
atv	106	9.7	9.7	107	9.6	9.6	213	19.3	19.3
hp-1	164	14.2	9.7	175	13.9	9.6	339	28.1	19.4
P value	0.237	0.003	0.466	0.248	0.006	0.201	0.243	0.004	0.315
Maturity Stage (B)									
Green	13.1	10.0	9.9	12.8	9.8	9.8	25.8	19.7	19.7
Breaker	411	14.2	9.8	429	13.8	9.8	839	28.1	19.6
Red	196	10.2	9.7	199	9.5	9.5	395	20.2	19.2
P value	<.0001	<.0001	0.826	<.0001	<.0001	0.212	<.0001	<.0001	0.541
(A) x (B) P value	0.025	0.007	0.020	0.028	0.007	0.007	0.027	0.006	0.010

Table 4. Total quercetin, kaempferol, and their ratio Q/K in fruit exocarp (peel), mesocarp (flesh), and seed cavity in different genotypes (A), fruit maturity stages (B), and their interaction [(A) x (B)].

	Total Quercetin ($\mu g\ g^{-1}$FW)			Total Kaempferol ($\mu g\ g^{-1}$FW)			Quercetin / Kaempferol		
Factors	Exocarp	Mesocarp	Seed Cavity	Exocarp	Mesocarp	Seed Cavity	Exocarp	Mesocarp	Seed Cavity
Genotypes (A)									
'Ailsa Craig'	1118	65.4	61.7	144	61.4	67.5 ab	7.7 a	1.1 a	0.91
aa	1137	81.8	57.8	142	64.2	66.8 a	7.6 a	1.3 a	0.87
atv	1110	63.4	56.6	131	61.7	70.6 ab	8.1 a	1.0 a	0.80
hp-1	2254	116	70.2	156	66.7	78.1 b	14.2 b	1.7 b	0.90
P value	0.001	0.001	0.153	0.419	0.341	0.035	<.0001	0.003	0.131
Maturity Stage (B)									
Green	1311	88.1	65.2	142	67.0	64.7 a	8.3 a	1.3 b	1.00 b
Breaker	1337	89.8	59.5	131	61.8	74.1 b	9.9 b	1.5 b	0.80 a
Red	1597	67.4	60.0	159	61.7	73.4 b	10.1 b	1.1 a	0.81 a
P value	0.163	0.001	0.870	0.006	0.104	0.004	0.043	0.001	0.015
(A) x (B) P value	0.007	0.027	0.866	0.014	0.318	0.302	0.113	0.280	0.617

[Z] Different letters indicate statistical differences. Mean separation by protected LSD (< 0.05)

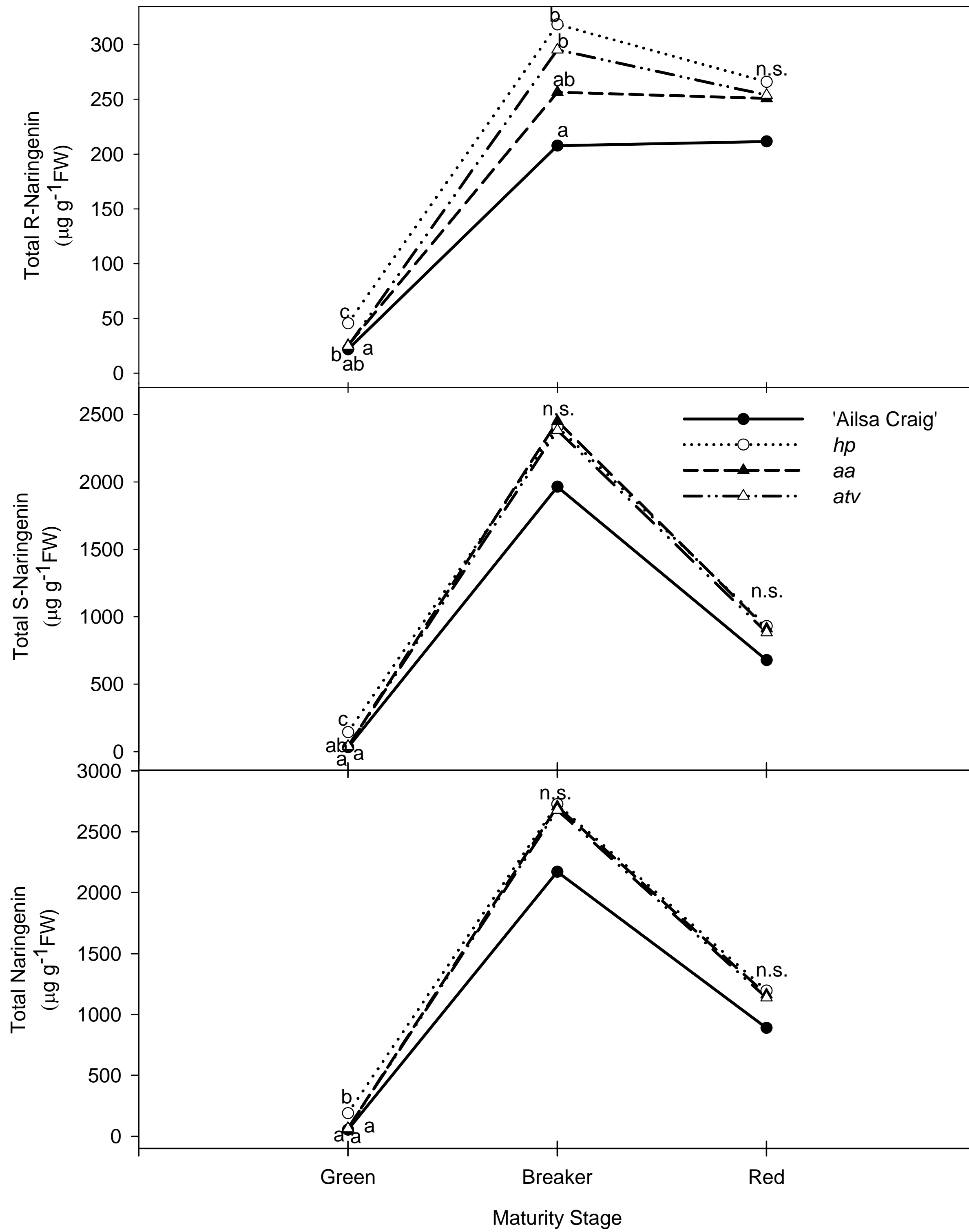


Figure 2

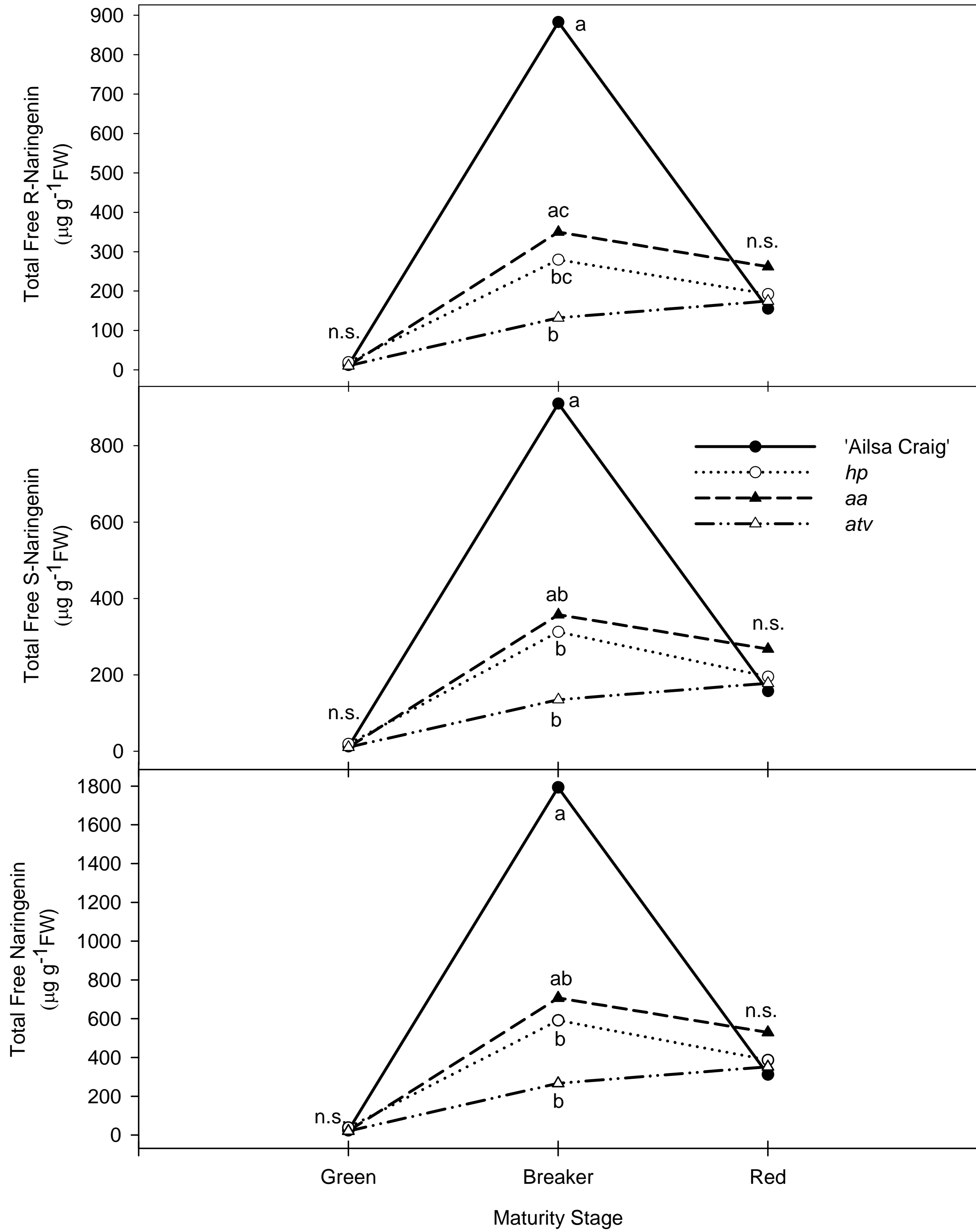


Figure 3

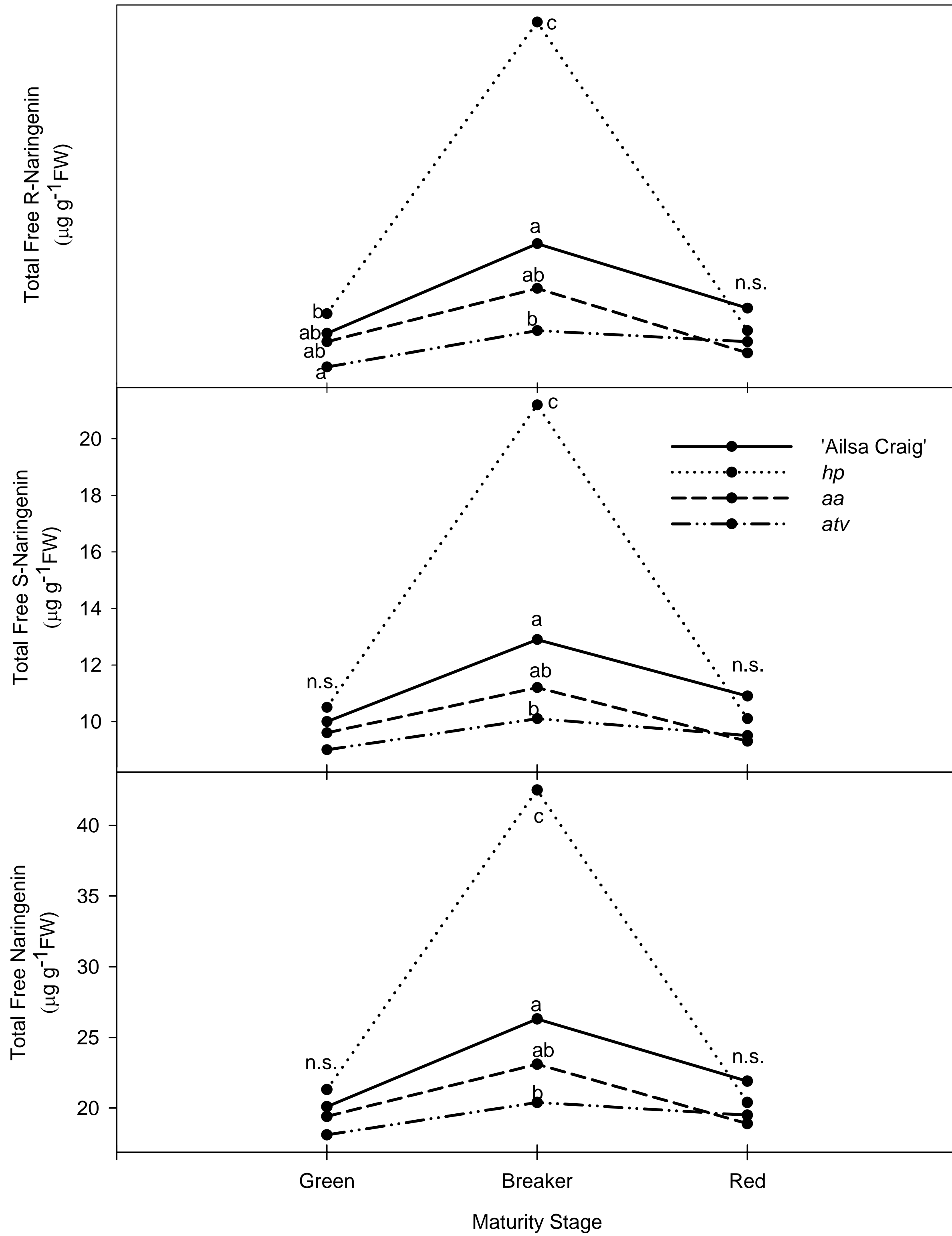


Figure 4

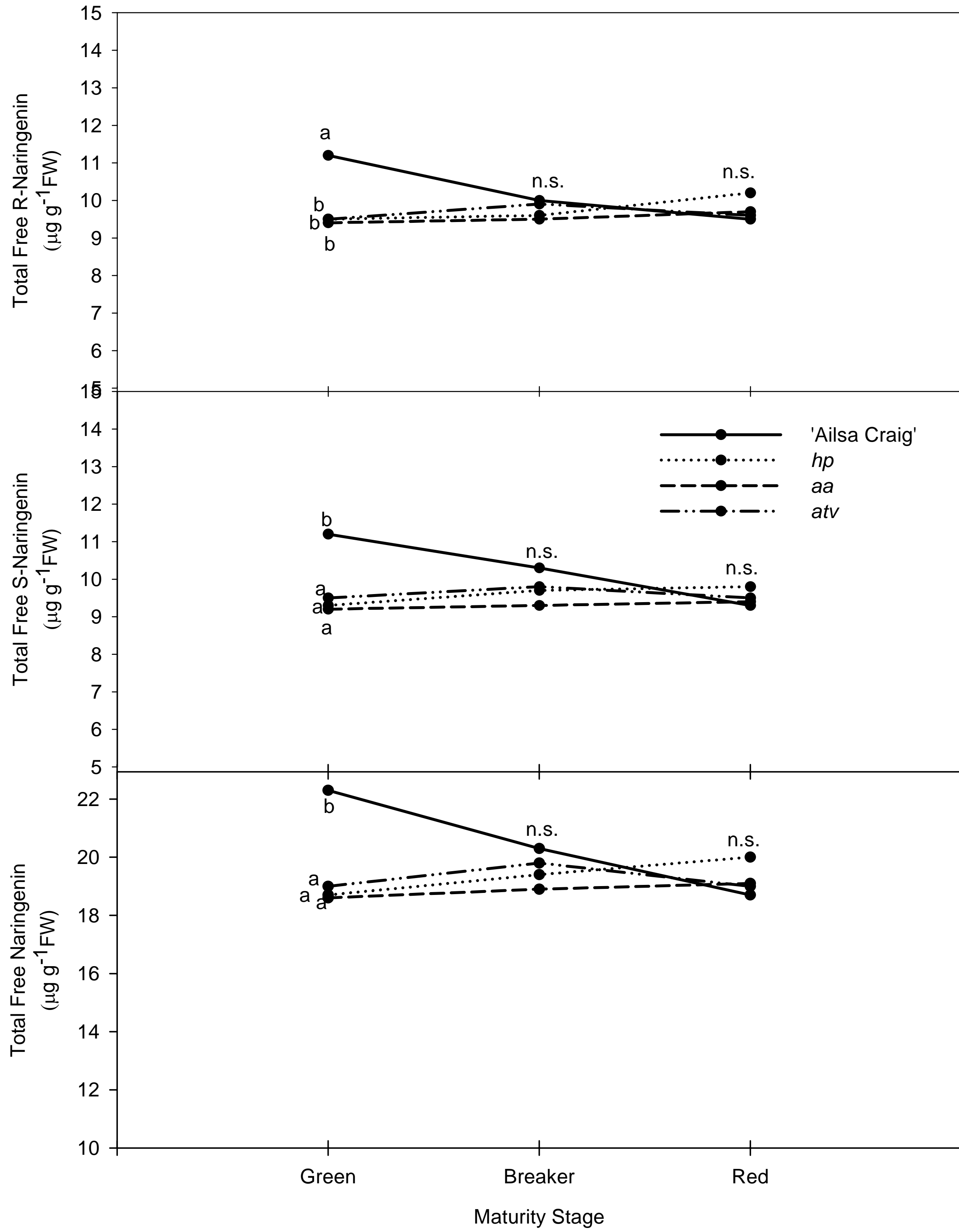


Figure 5

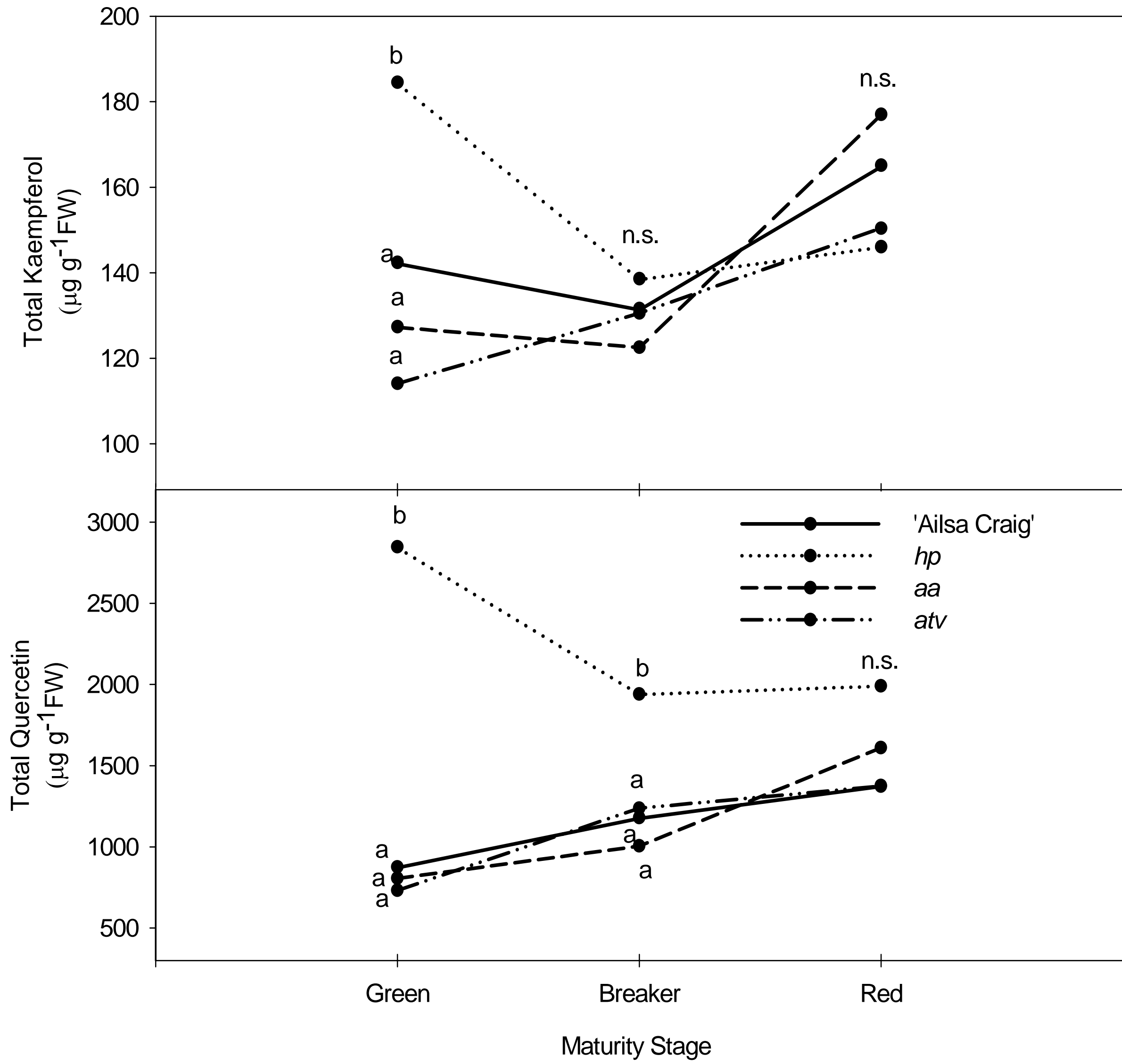


Figure 6

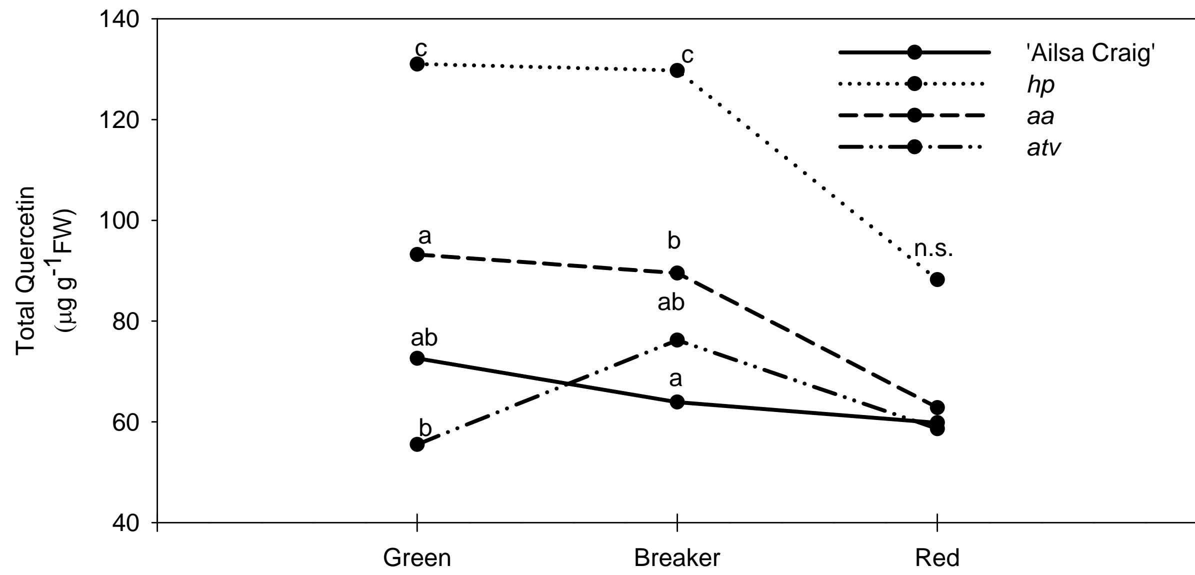


Figure 7

CHAPTER 5

CONCLUSIONS AND GENERAL DISCUSSION

Photooxidative damage of fruit or sunscald is a photodynamic injury caused by high light and elevated temperature conditions. The observed symptoms vary among cultivars and species, but they always involve discoloration and/or bleaching of the surface of fruit exposed to direct sunlight. Symptoms of sunscald appear both on fruit acclimated to high solar radiation and on non-acclimated fruit that are suddenly exposed. The photodynamic reactions in chlorophyll-containing tissues lead to increases in reactive oxygen species (ROS) as a consequence of a decrease in efficiency of PSII. In Chapter 1 I reviewed in detail the environmental factors affecting sunscald of fruit, species susceptibility, biochemical factors that affect tissue susceptibility, and acclimation directly related to this photooxidative stress, and summarized our current knowledge of photooxidative damage in fruit. Some of the conclusions from this review were that photooxidative stress in fruit is believed to be similar to this stress in leaves, but there is still very little supporting evidence. Clearly, both elevated temperatures and high direct solar radiation are critical for inducing sunscald damage. However, there is no conclusive evidence establishing the role of UV radiation in sunscald development. There is also insufficient information addressing cellular or macromolecular damage of affected tissues. In addition, there is limited or incomplete information on the response of antioxidant systems

during sunscald events. There is also conflicting information on ascorbate levels, but exposed fruit affected by sunscald have shown higher amounts of total flavonoids and phenolics than shaded fruit. Finally, only a few papers have addressed specific antioxidant enzymes, such as superoxide dismutase, but none have described the whole antioxidant system.

In Chapters 2 and 3, I presented the first thorough study of physiological and biochemical factors involved in photooxidative injury or sunscald development in fruit. This was achieved after developing a system that permitted the imposition of short-term photooxidative stress on detached, immature-green tomato (*Lycopersicon esculentum* Mill) fruit under natural solar radiation. Seven genotypes, including mutants with attenuated or enhanced specific antioxidant components, were tested. In general, the responses of fruit exocarp to photooxidative stress were independent of genotype, implying that physiological and biochemical responses, but not genetic factors, were strongly associated in these tomato genotypes. Therefore, tomato fruit could be considered a functional model for other species whose fruit are severely affected by sunscald, such as apples.

The photooxidative stress treatments applied to detached tomato fruit reproduced sunscald symptoms observed on fruit attached to the plant. Both high temperature and solar irradiance caused fruit surface discoloration with faster degradation of chlorophyll (Chl) than carotenoids (Car), leading to an increase in the Car/Chl ratio. Bleaching of the fruit surface was mostly caused by solar irradiance, whereas high temperatures were responsible for most inactivation of

photosynthesis, measured as a decrease in F_v/F_m. Among flavonoids, quercetin and kaempferol concentrations increased rapidly upon exposure to sunlight, but they were not affected by natural ultraviolet (UV) radiation, suggesting rapid photo-protection in response to visible light. Naringenin synthesis was not induced under the high light conditions of this study suggesting a different function than photoprotection for this flavonoid. The ascorbate and glutathione pools and total soluble protein in fruit exocarp decreased as duration of exposure increased. The specific activities of the antioxidant enzymes (SOD, APX, DHAR, MDHAR, GR, CAT) increased rapidly in exocarp cells, as well as the amount of some of those enzymes (MDHAR and GR) as a fraction of total soluble protein. This appeared to be the first phase of acclimation to the imposed environmental conditions. UV radiation partially stimulated the activities of APX, MDHAR, and CAT. Together, these mechanisms appeared to prevent the accumulation of lipid peroxidation products and perhaps also DNA damage and protein oxidation products.

The knowledge gained from the experiments described in Chapters 2 and 3 provided an opportunity to better describe the events of photooxidative stress in fruit. Figure 5-1 summarizes and integrates this knowledge and presents these processes beginning with the induction of photooxidative stress by visible solar irradiance. Visible light appears to be the environmental factor most responsible for common sunscald symptoms and the “static” and “dynamic” biochemical responses that provide the protective mechanisms against photooxidative stress. As photooxidative stress progresses, photoinhibition of chlorophyll-containing

fruit tissues and ROS production increases. This oxidative stress then stimulates responses from all antioxidant systems. Antioxidant metabolites, such as ascorbate and glutathione, decrease initially, but antioxidant enzyme activities increase. If fruit are unable to acclimate to the environmental stress, they eventually suffer damage to macromolecules, with visible and eventually severe sunscald symptoms appearing.

In Chapters 2 and 3, I also show that fruit exocarp from the *high pigment*-1 (*hp-1*) mutant had higher Chl and Car levels than the other genotypes, and more kaempferol and quercetin than its parent ‘Ailsa Craig’. However, its photosynthetic efficiency was similar to the other genotypes. *Hp-1* appears to have greater tolerance to photooxidative stress as a consequence of its significantly higher ascorbate content and APX and GR activities, which allow the fruit of this mutant to better cope with increasing ROS production as a consequence of imposed photooxidative stress.

Finally, in Chapter 4 I present an extensive study of naringenin enantiomers, and quercetin and kaempferol contents, among different tomato fruit tissues (exocarp, mesocarp, seed cavity) at different maturity stages (green, ”breaker”, ripe-red) in three mutants (*anthocyanin abscent, atroviolacea,* and *hp-1*) and their nearly isogenic parent (‘Ailsa Craig’). In this portion of the study, I used a procedure to determine the total amounts of these flavonoids by removing their attached sugar moieties using enzymatic hydrolysis, and a new stereospecific HPLC procedure to separate naringenin enantiomers. The enzymatic hydrolysis with β-glucuronidase allowed for quantification of greater

amounts of these flavonoids when compared with the published literature. Glycosylated S-naringin was the predominant enantiomer in all fruit tissues, although free R- and S-naringenin were detected in both exocarp and mesocarp tissues. While there was more quercetin than kaempferol in exocarp tissue, they were present in about equal concentrations in the mesocarp. Quercetin concentrations were higher in the exocarp and mesocarp of immature green and “breaker” fruit of the *hp-1* mutant than in the other genotypes. These results again demonstrate the more favorable potential antioxidant capacity of this mutant, and suggest its future use for studying photooxidative stress in fleshy fruit and as breeding material for protection against sunscald in tomato.

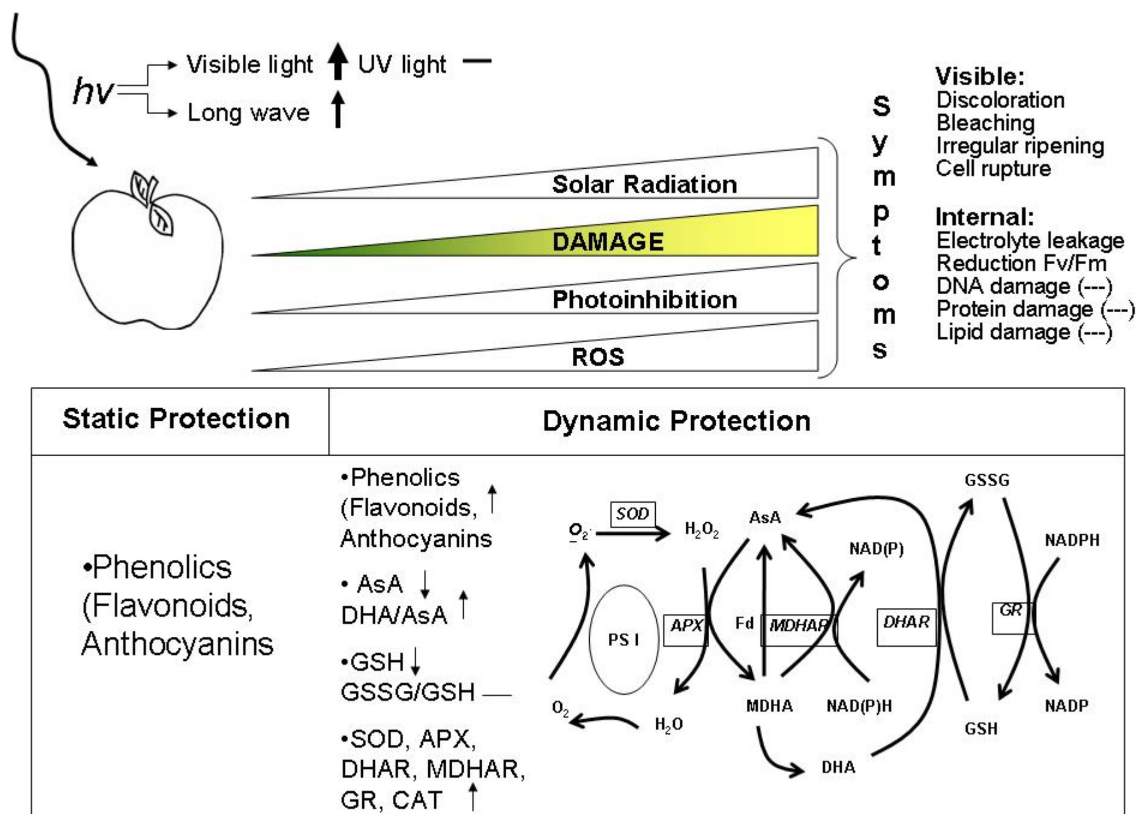


Figure 5.1. Environmental (solar radiation), physiological (photosynthetic efficiency), and biochemical factors, representing both "static" and "dynamic" mechanisms of protection involved in photooxidative damage in fleshy fruits.