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RESEARCH PAPER

Physiological and biochemical responses of fruit exocarp of tomato (*Lycopersicon esculentum* Mill.) mutants to natural photo-oxidative conditions

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Abstract

Photo-oxidative stress was imposed under natural solar radiation on exposed and shaded sections of detached fruit of immature green tomato (*Lycopersicon esculentum* Miller=*Solanum lycopersicum* L.) mutants (*anthocyanin absent*, β-*carotene*, *Delta*, and *high pigment-1*) and their nearly isogenic parents ('Ailsa Craig' and 'Rutgers'). After 5 h exposure to high solar irradiance, either with or without ultraviolet (UV) radiation, surface colour changes, pigment composition, photosynthetic efficiency, antioxidant metabolites and enzyme activities, and selected flavonoids and antioxidant proteins in exocarp tissue were evaluated. The imposed photo-oxidative stress reproduced the symptoms 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. Surface bleaching was mostly caused by visible light, whereas elevated temperatures were mostly responsible for the inactivation of photosynthesis, measured as decreased F_v/F_m. Ascorbate, glutathione, and total soluble protein concentrations decreased in the exocarp as the duration of exposure increased. Specific activities of superoxide dismutase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and catalase increased with exposure, suggesting that these proteins were conserved during the imposed stress. GR protein expression remained stable during the imposed stress, whereas, MDHAR protein expression increased. Quercetin and kaempferol concentrations increased rapidly upon exposure, but not to UV radiation, suggesting rapid photo-protection in response to visible light; however, naringenin synthesis was not induced. The apparent increased tolerance of *hp-1* fruit is discussed.

Key words: Acclimation, antioxidant, fruit, oxidative stress, photo-oxidation, photosynthesis, sunscald, UV.

Introduction

In fruit, photo-oxidative damage is typically due to photodynamic injury of heated tissue that occurs under conditions of intense sunlight and elevated temperatures (Barber and Sharpe, 1971). This form of photo-oxidative damage, commonly called 'sunscald', can be severe enough to cause economic losses in tomatoes (Moore and Rogers, 1943; Ramsey *et al*., 1952; Retig and Kedar, 1967; Rabinowitch *et al*., 1974) and apples (Brooks and Fisher, 1926; Moore and Rogers, 1943; Andrews and Johnson, 1996; Yuri *et al*., 1996), and other fruit crops.

In tomato (*Lycopersicon esculentum* Miller=*Solanum lycopersicum* L.) fruit, photo-oxidative damage occurs mainly in the green epidermal and hypodermal tissues, although 'mature-green' and 'breaker' fruit are the most susceptible (Retig and Kedar, 1967). Photo-oxidative stress in fruit is linked with fruit maturation, because oxidative processes have been implicated in the ripening process (Blackman and Parija, 1928; Brennan and Frenkel, 1977; Jiménez *et al*., 2002; Andrews *et al*., 2004).

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Although both high irradiance and elevated temperatures are necessary to cause 'sunscald' symptoms, there has been little research addressing the importance of these environmental factors individually, as well as the role of ultraviolet (UV) radiation on photo-oxidative injury of fruit.

Photo-oxidative damage is caused by reactive oxygen species (ROS), the flux of which are greatly increased under photo-oxidative stress (Foyer *et al*., 1994). ROS, produced normally and continuously by cells, are tightly controlled by complex and dynamic antioxidant systems. Plant antioxidant defences 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. Isoenzymes of superoxide dismutase (SOD; EC 1.15.1.1) catalyse the dismutation of the superoxide anion ($O_2^{\cdot -}$), producing hydrogen peroxide (H_2O_2), which is reduced to water by either catalases (CAT; EC 1.11.1.6) (Willekens *et al*., 1997) or ascorbate peroxidases (APX; EC 1.11.1.11). APXs utilize reduced ascorbic acid (AsA) as an electron donor (Groden and Beck, 1979; Anderson *et al*., 1983). Monodehydroascorbate (MDHA), an oxidized form of AsA, is reduced to AsA via reduced ferredoxin (Miyake and Asada, 1994) or by the ascorbate–glutathione cycle (Foyer and Halliwell, 1976) via monodehydroascorbate reductase (MDHAR; EC 1.6.5.4). Dehydroascorbate (DHA), another oxidized form of AsA is converted back to AsA by dehydroascorbate reductases (DHAR; EC 1.8.5.1), which utilize glutathione (GSH) as reductant (Foyer *et al*., 1994). GSH is regenerated by glutathione reducatse (GR; EC 1.6.4.2) from its oxidized form, glutathione disulphide (GSSG).

Among fruit crops, tomatoes serve as model species for understanding fruit physiology, biochemistry, and genetics. In the present study, tomato fruit was used to study physiological and biochemical components involved in photo-oxidative damage. To accomplish this, a system utilizing detached fruit and natural solar radiation to accelerate injury was implemented. Tomato mutants were also selected with attenuated/enhanced pigments and/or antioxidant metabolites to evaluate the importance of certain antioxidant components on fruit acclimation.

Materials and methods

Tomato mutants

Seven tomato (*L. esculentum* Mill.) genotypes were used in this study. In the first, *anthocyanin absent* (*aa*) (LA 3617), with a mutation in chromosome 2, anthocyanin is completely absent in all plant parts (CM Rick Tomato Genetics Resources Center; University of California, Davis, CA, USA). The second mutant, β-*carotene* (*B*) (LA 3179), with a mutation at loci 6 and 106 (Stevens and Rick, 1986), has high β-carotene and low lycopene concentrations in bright orange mature fruit (Tomes *et al*., 1956, 1958; Harris and Spurr, 1969; Ronen *et al*., 2000). 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). The third mutant, *Delta* (*Del*) (LA 2996A), has inhibited lycopene and increased δ-carotene (Williams *et al*., 1967) in reddish-orange mature fruit. The fourth mutant, *high pigment* (*hp-1*), represented by two genotypes (LA 2838A, *hpA*, and LA 3004, *hpR*), has 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 (Thompson, 1955, 1961; Baker and Tomes, 1964; Clayberg *et al*., 1970; Jarret *et al*., 1984; Stevens and Rick, 1986; Torres, 2001; Andrews *et al*., 2004), as well as anthocyanins (Wettstein-Knowles, 1968*a*, *b*; Kerckhoffs *et al*., 1997). The mutation is located on a negative regulator(s) of phytochrome signal translation, which causes amplified photo-responsiveness and pleitropic effects (Kerr, 1965; Jarret *et al*., 1984; Kerckhoffs *et al*., 1997; Peters *et al*., 1998). All mutants are nearly isogenic in the cultivar 'Alisa Craig' (AC), except LA 3004, which is nearly isogenic in 'Rutgers' (R).

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

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–September) 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 germinated in cell packs in a greenhouse and grown until they had several true leaves, at which time they were transplanted in a replicated, randomized complete block design in the field, consisting of four 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, 1 h d^{-1} during the first 30 d after planting, and 2 h d^{-1} from 30 d after planting until the end of the growing season. The output of each emitter was 1.9 l h^{-1}. Plants were fertilized 2 weeks after transplanting with calcium nitrate (30 g per plant).

Experimental set-up

Determination of susceptibility to photo-oxidative 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 thick safety glass plate filter), for 0, 2.5, and 5 h exposure. In order to investigate the independent effects of solar radiation and high temperature on photo-oxidative injury, half of each fruit from the treatments previously described was covered with reflective tape allowing air movement on the fruit surface beneath.

Each treatment was represented by a group of four similar-sized fruits, harvested from non-sunlit 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. The set of treatments described previously were replicated on five separate dates in 2003: 17, 22, and 29 July and 13 and 26 August. On all dates, experiments were conducted between 11.30 h and 17.00 h 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 photo-oxidative stress was $(F_m–F_0)/F_m$ or F_v/F_m, where F_0 and F_m are the minimal and maximal fluorescence yield of a dark-adapted measurement. By convention, F_v is variable fluorescence, $F_m–F_0$. 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 experimental date.

Surface colour changes were measured using a colourimeter (Minolta CR-300, Ramsey, NJ, USA). Data were expressed in CIELAB units where L^* indicates lightness/darkness, and a^* blue-green/red-purple and b^* yellow-blue hue components. The a^* and b^* coordinates were used to determine hue angle ($\tan^{-1}$ (b^*/a^*), 0°–360°) or colour, and chroma [C, $(a^{*2}+b^{*2})^{1/2}$] or colour saturation or intensity (McGuire, 1992). Measurements were taken in triplicate per fruit on the treated fruit surface on all experimental dates.

Biochemical assays

Sampling: The exocarp (up to 2 mm thick) was removed separately with a scalpel from the two sections, exposed and covered with reflective tape, from fruit submitted to the treatments. 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.

Pigments: Chlorophyll (Chl *a* and Chl *b*) and total carotenoids (Car) were extracted from 50–150 mg of frozen exocarp tissue in 1 ml of 100% acetone at −20 °C for 24 h. Extracts were then centrifuged at 5000 *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):

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

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

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

Antioxidant metabolites: Approximately 0.2 g of exocarp tissue per sample was extracted by grinding in liquid N_2, acid-washed sand, and 1.5 ml of 1 M $HClO_4$. Following centrifugation (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 or 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, as before, 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 reaction mixture containing 0.1 M Na_2HPO_4 buffer (pH 5.6) and sample extract (Andrews *et al*., 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 buffer (pH 7.5). Reduced glutathione (GSH) and its oxidized form (GSSG) were measured spectrophotometrically by the modified methods of Griffiths (1980) via a kinetic reaction at 412 nm by adding 1.0 unit glutathione reductase (from Bakers yeast, Sigma-Aldrich, USA) to a reaction mixture containing 0.1 M Na_2HPO_4 buffer (pH 7.5), 6 mM EDTA, 6 mM 5–5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), 10 mM NADPH, and 10 µl sample extract.

Antioxidant enzymes: Enzymes were extracted by grinding ~0.2 g frozen exocarp in liquid N_2, acid-washed sand, 50 mM MES/KOH buffer (pH 6.0), 40 mM KCl, 2 mM $CaCl_2$, and 1 mM L-AsA. After centrifugation (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 of samples. All enzyme activity assays were conducted at 20 °C in 0.5 ml reaction volume.

SOD activity was assayed as described by McCord and Fridovich (1969) with some modifications in a reaction mixture of 50 mM HEPES buffer (pH 7.8), 0.5 mM EDTA, 0.5 mM nitroblue tetrazolium, 4 mM xanthine, 50 µl extract, and 0.04 units xanthine oxidase. After 10 min, absorbance was measured at 560 nm. SOD activity was determined by a standard curve using horseradish SOD (Sigma-Aldrich, St Louis, MO, USA).

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

MDHAR activity was measured in a reaction mixture of 100 mM HEPES buffer (pH 7.6), 2.5 mM L-AsA, 250 µM NADH, and 10 µl extract, with 0.4 units of ascorbate oxidase added to start the reaction. DHAR activity was determined in a reaction mixture of 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, and 10 µl extract, with 0.2 mM DHA added to initiate the reaction. Change in absorbance was monitored for 3 min at 340 or 265 nm and activity was calculated from this reaction rate using extinction coefficients of 3.3 mM^{-1} or 7.0 mM^{-1} for MDHAR and DHAR, respectively (Miyake and Asada, 1992).

GR was measured in a mixture of 50 mM HEPES buffer (pH 8.0), 0.5 mM EDTA, 250 µM NADPH, and 10 µl extract, with non-enzymatic NADPH oxidation measured in each reaction mixture before 500 µM GSSG was added to start the reaction (Foyer and Halliwell, 1976). Change in absorbance was monitored for 3 min at 340 nm and 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 and Maehly (1955) in a reaction mixture containing 50 mM KH_2PO_4 buffer (pH 7.0), 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).

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 loading buffer consisting of 50 mM TRIS–HCl (pH 7.3), 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 7000 *g* for 5 min at 25 °C before loading.

Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) using an electrophoretic transfer cell (Model Mini-Trans-Blot, Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 1 h, and probed with polyclonal antibodies, rabbit anti-*Zea mays* leaf MDHAR (Ushimaru *et al*., 1997) diluted 1:1000 and rabbit anti-spinach leaf GR (Tanaka *et al*., 1994) diluted 1:4000. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories,

Hercules, CA, USA) 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).

Flavonoids: Flavonoids were extracted from frozen tissue with methanol and hexane, reconstituted in the mobile phase, and enzymatically hydrolysed with *Helix pomatia* Type-HP-2 β-glucuronidase to measure total quercetin, kaempferol, and naringenin enantiomers (Torres *et al*., 2005; Yañez and Davies, 2005). Daidzein and 7-ethoxycoumarin were added as internal standards for naringenin, and quercetin and kaempferol, respectively. Extracts with 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×4.5 mm I.D., 5 μm particle size; Chiral Technologies Inc., Exton, PA, USA) using an isocratic mobile phase of acetonitrile: water:phosphoric acid (30:70:0.04 by vol.) at a flow rate of 0.4 ml min^{-1} at 25 °C, with detection at 292 nm. Quercetin and kaempferol were also separated isocratically by a Chiralcel AD-RH column (150 mm×4.5 mm ID, 5 μm particle size; Chiral Technologies Inc., Exton, PA, USA), using a mobile phase of acetonitrile:water:phosphoric acid (42:58:0.01 by vol.) at a flow rate of 0.6 ml min^{-1}, with detection at 370 nm.

Statistical analysis

The experimental design was analysed as a split-split block with three factors as main effects: (i) genotype (A), (ii) duration of exposure (B), and (iii) presence or absence of UV radiation (C). The interactions of these three factors are indicated in the tables as (A)×(B), (A)×(C), (B)×(C), and (A)×(B)×(C). Covered and exposed sections of fruit were analysed separately. 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 Bonferroni 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 the concentration of the standards. *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

The photosynthetic photon flux density (400–700 nm) and UV radiation (295–385 nm) were measured in ambient sunlight and under the glass filter on each sampling date with quantum sensors (Li-190SA, Li-Cor, Lincoln, NE, USA) and a total UV radiometer (TUVR, The Eppley Laboratory, Newport, RI, USA), respectively. The glass filter (−UV) reduced UV radiation by ~95%, as well as *PPFD* by ~20% (Torres, 2005). The surface temperatures of exposed fruit, measured with copper-constantan thermocouples connected to a datalogger (Model CR10X, Campbell Scientific, Logan, UT, USA), averaged 12 °C higher than air temperature (Torres, 2005). Surface temperatures of the exposed portion of fruit and the section shaded by reflective tape showed a similar diurnal pattern, but temperatures of the exposed surface of fruit not under the glass filter were ~3–4 °C higher than the exposed fruit surface under the glass (Torres, 2005).

Surface colour and pigments

As duration of natural sunlight exposure increased, the fruit surface became less green and more yellow, measured as a decrease in hue angle (Table 1). This discoloration mainly occurred during the first 2.5 h of exposure. Fruit colour also became less saturated or intense (i.e. duller) as time passed, indicated by a decrease in C values (Table 1). When genotypes were compared, both *hp-1* (*hpA* and *hpR*) mutants had the same hue or green colour as the parents and other mutants, but their colour was significantly intensified (higher C) and darker (smaller *L**) than the parents (Table 1). This was observed after either 2.5 or 5.0 h of exposure ($P=0.002$). No significant differences were detected in any colour parameter between fruit fully exposed to sunlight (+UV) or under the glass filter (−UV). When the effect of temperature without direct sunlight was analysed by comparing covered and exposed sections of fruit, the exposed section had lower hue and C values, indicating greater discoloration and less colour intensity, respectively, after 2.5 h (data not shown) and 5 h of exposure (Table 2). There was also a slight lightening (larger *L**), representing chlorophyll bleaching, in surface colour of the exposed section compared with the covered section of fruit at 2.5 h (data not shown) and 5 h of exposure (Table 2). Although non-significant, *L** showed an inverse linear trend as duration of exposure increased (Table 1), suggesting chlorophyll bleaching.

Changes in pigment concentrations in fruit exocarp confirmed colour measurements and observed visible symptoms. As duration of exposure increased, Chl concentrations decreased (Table 3). Chl *b* was degraded faster than Chl *a*, represented by an increase in Chl *a*/Chl *b* ratio with exposure (Table 3). Total Car decreased, but only after 5 h of exposure (Table 3). The Car/Chl ratio increased with exposure (Table 3) concomitant with the change in fruit colour from green to yellow (i.e. decreasing hue angle) (Table 1).

The glass filter, which attenuated UV radiation, did not significantly affect pigment composition of fruit exocarp with the exception of Chl *b*, which increased slightly under −UV radiation (Table 3). For total Chl and Car concentrations of exposed fruit, there was a significant interaction between duration of exposure and UV radiation (Table 3). There were significant reductions in both Chl and Car concentrations in UV-exposed (+UV) exocarp after 5 h of exposure (data not shown).

Fruit exocarp from both *hp-1* mutants (*hpA, hpR*) had 3–5-fold higher Chl and Car levels compared with the

Table 1. *Surface colour 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 Bonferroni adjustment.

Factors	L*	C	Hue (°)
Genotypes (A)			
'Ailsa Craig'	65.2 ab[a]	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	<0.0001	<0.0001	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	<0.0001
P-value, non-linear	0.954	0.466	0.003
(A)×(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)×(C) *P*-value	0.259	0.920	0.370
(B)×(C) *P*-value	0.512	0.175	0.237
(A)×(B)×(C) *P*-value	0.489	0.715	0.760

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

Table 2. *Surface colour parameters (lightness, L*; chroma, C; and hue angle), pigment concentrations (chlorophyll; Chl* a*, Chl* b*, total Chl; carotenoids, Car), chlorophyll fluorescence (*F_v/F_m*), and flavonoids (kaempferol, K; quercetin, Q; Q/K ratio) from sunlight-exposed and covered sections of immature green fruit after 5 h of exposure*

P-value indicated for comparison within rows.

Variable	Exposed	Covered	*P*-value
Colour parameters			
L*	63.9	63.3	0.073
C	30.1	34.1	<0.0001
Hue (°)	108.1	111.2	<0.0001
Pigments			
Chl *a* (μg g^{-1} FW)	24.4	35.7	<0.0001
Chl *b* (μg g^{-1} FW)	3.4	6.8	<0.0001
Total Chl (μg g^{-1} FW)	27.7	42.5	<0.0001
Car (μg g^{-1} FW)	8.9	11.8	0.006
Chl *a*/Chl *b* ratio	9.2	6.1	<0.0001
Car/Chl ratio	0.38	0.31	0.001
Chl fluorescence			
F_v/F_m	0.19	0.31	0.001
Flavonoids			
K (μg g^{-1} FW)	236.5	201.7	0.115
Q (μg g^{-1} FW)	3051.2	1268.2	0.032
Q/K ratio	12.3	5.9	0.024

other genotypes (Table 3). *HpA* was the only genotype whose Car/Chl ratio was lower than its parent (Table 3). The covered section of exocarp had higher Chl (*a* and *b*) and Car concentrations, but lower Chl *a*/Chl *b* and Car/Chl ratios, than the exposed section after both 2.5 h (data not shown) and 5 h of exposure (Table 2). 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 3). Chl *b* concentrations in exocarp from the exposed section of fruit varied with genotype (Fig. 1).

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 4). F_v/F_m, or intrinsic PSII 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 exposed 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 (data not shown) and 5 h of exposure (Table 2). 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. 2).

Antioxidant metabolites

Reduced AsA, as well as the redox ratio [AsA/(AsA+DHA)], decreased significantly in the exocarp of immature green fruit as duration of exposure increased (Table 5). The rate at which AsA decreased was greater than the rate at which DHA decreased, which is evident by the increase in the DHA/AsA ratio as the duration of exposure increased (Table 5). 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 respond quickly to the imposed photo-oxidative stress (Table 5). By 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 5). AsA and total ascorbate (AsA+DHA) 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 5).

The glass filter, which removed 95% of UV radiation, had no effect on ascorbate levels in the exocarp of immature green fruit (Table 5). Fruit of both *hp-1* mutants (*hpA* and *hpR*), but especially *hpA*, contained significantly higher concentrations of both AsA and DHA than the other

Table 3. *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 Bonferroni adjustment.

Factors	Chl *a* (μg g^{-1} FW)	Chl *b* (μg g^{-1} FW)	Total Chl (μg g^{-1} FW)	Total Car (μg g^{-1} FW)	Chl *a*/Chl *b* ratio	Car/Chl ratio
Genotypes (A)						
'Ailsa Craig'	17.3 a[a]	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	<0.0001	<0.0001	<0.0001	<0.0001	0.1482	0.001
Duration (B)						
0 h	39.1	7.57	46.6	11.1	5.5	0.25
2.5 h	36.4	5.00	41.4	12.5	12.9	0.35
5 h	24.4	3.37	27.7	8.9	9.2	0.38
P-value, linear	<.0001	<.0001	<.0001	0.001	0.005	<.0001
P-value, non-linear	0.360	0.290	0.710	0.001	<.0001	0.005
(A)×(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)×(C) *P*-value	0.455	0.408	0.426	0.167	0.525	0.069
(B)×(C) *P*-value	0.277	0.055	0.018	0.021	0.036	0.779
(A)×(B)×(C) *P*-value	0.332	0.201	0.061	0.170	0.422	0.058

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

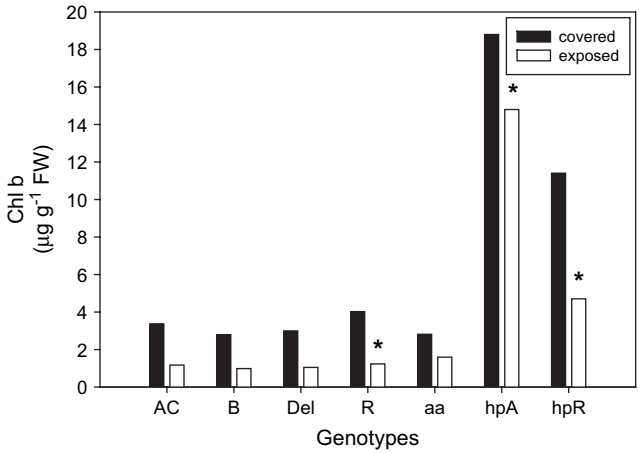

Fig. 1. Effect of genotype and exposure (covered or exposed) on chlorophyll (Chl) *b* concentration in exocarp from immature green fruit after 5 h of exposure to sunlight. An asterisk indicates statistically different covered versus exposed within genotypes (Tukey HSD, P <0.05).

genotypes, but their redox and DHA/AsA ratios did not differ (Table 5). When exposed and covered sections of fruit were compared, exocarp from the sunlight-exposed section had less total ascorbate than exocarp from the covered section after 5 h of exposure (Table 6). Although differences in AsA and DHA concentrations between exposed and covered sections of the fruit were non-significant, both declined sufficiently to result in a significant decrease in total ascorbate from the imposed photo-oxidative stress.

Both GSH and total glutathione (GSH+GSSG) concentrations decreased linearly in fruit exocarp as duration of exposure increased (Table 7). Neither the redox ratio [(GSH/(GSH+GSSG)] nor the GSSG/GSH ratio changed with duration of exposure (Table 7). Just as for ascorbate, UV radiation did not significantly affect glutathione levels in fruit exocarp (Table 7). There were also no significant differences in glutathione concentrations or ratios among genotypes (Table 7), or between sections of fruit that were covered or exposed to solar irradiance (Table 6).

Antioxidant enzymes

When enzyme activities were expressed on a fresh weight (FW) basis, different trends were observed as duration of exposure increased (Table 8). 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 8). Only GR and CAT activities were affected by exposure to UV radiation, with CAT activity more than doubling but GR activity decreasing (Table 8). Exocarp from *hpA* fruit had the highest APX and GR activities among genotypes (Table 8). APX, GR, and CAT activities expressed on a FW basis were higher in the exocarp of the covered section compared

Table 4. 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 Bonferroni 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 (B)	
0 h	0.71
2.5 h	0.18
5 h	0.19
P-value, linear	<0.0001
P-value, non-linear	<0.0001
(A)×(B) *P*-value	0.389
UV radiation (C)	
+UV	0.37
−UV	0.37
P-value	0.896
(A)×(C) *P*-value	0.206
(B)×(C) *P*-value	0.012
(A)×(B)×(C) *P*-value	0.210

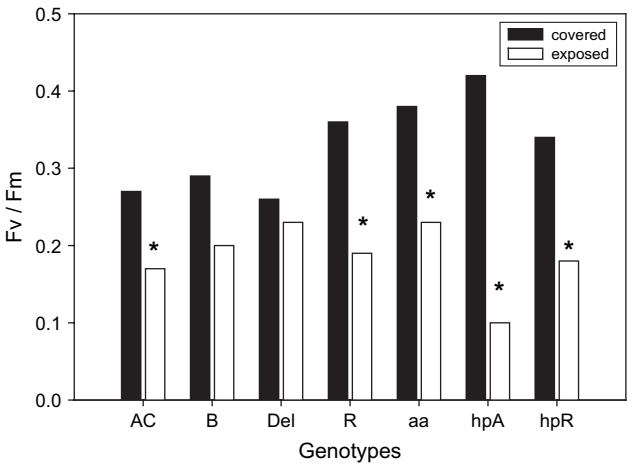

Fig. 2. Effect of genotype and exposure (covered or exposed) on F_v/F_m ratio in exocarp from immature green fruit after 2.5 h of exposure to sunlight. An asterisk indicates statistically different between covered and exposed within genotypes (Tukey HSD, P <0.05).

with the section exposed to solar irradiance (Table 9), however, when expressed on a protein basis all enzyme activities except GR and CAT were higher in the exposed section (Table 9).

Exocarp of immature fruit of both *hp-1* mutants had the highest protein concentrations compared with their parents and the other mutants, except the *aa* mutant (Table 10). Protein concentrations decreased linearly as duration of exposure increased (Table 10). After 5 h of exposure to solar irradiance there was only half of the initial protein concentration in the exocarp (Table 10). The protein concentration was also lower in the exocarp from sections of fruit exposed to sunlight compared with sections of fruit that were covered (Table 9). UV radiation apparently 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 (Table 10).

Exocarp of *hpR* had the lowest APX activity per unit protein, but it was not significantly different from AC, *hpA*, and *aa* (Table 10). By contrast, *Del* had much higher CAT specific activity than the other genotypes (Table 10). Although non-significant, SOD, MDHAR, and GR activities of *Del* exocarp appeared to be higher, this partially may be due to its lower total protein concentration. All enzyme activities increased per unit protein with duration of exposure, but this was partly due to reduced protein concentration (Table 10). 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 the section of fruit exposed to UV radiation compared with the unexposed section (Table 10). For all enzymes, except CAT, specific activities were significantly higher in exocarp from the sunlight-exposed section of fruit than from the covered section (Table 9).

SDS-PAGE immunoblotting confirmed that the expression of MDHAR protein in the exocarp of immature green fruit increased from 0 h and 2.5 h to 5 h of sun exposure, while GR protein expression remained stable (Fig. 3).

Flavonoids

Specific flavonoids were only studied in the most apparently tolerant genotype to photo-oxidative stress, *hpA*, and its parent 'Ailsa Craig' (AC). Kaempferol and quercetin concentrations were significantly higher in *hpA* fruit exocarp than AC (Table 11). No differences were found in quercetin or kaempferol concentrations, or Q/K ratio, as duration of exposure increased (data not shown). 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 did not result in significant differences in flavonoid concentrations or ratios, despite the apparent higher concentrations in −UV exocarp (data not shown).

Only quercetin concentration and Q/K ratio were higher in the exocarp of the sunlight-exposed section compared with the covered section after 5 h of exposure (Table 2). The concentrations of both the *R*- and *S*-naringenin enantiomers were significantly higher in the exocarp of the *hpA* mutant than in AC (Table 11). *HpA* exocarp had a larger proportion of *S*-naringenin than AC, represented by

Table 5. *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 different tomato genotypes (A), duration of exposure to natural sunlight (B), and presence or absence of ultraviolet (+UV or −UV) radiation (C) on exposed sections of immature green fruit*

Trend contrasts *P*-values represent a Bonferroni adjustment.

Factors	AsA (μmol g^{-1} FW)	DHA (μmol g^{-1} FW)	AsA+DHA (μmol g^{-1} FW)	Redox ratio (AsA/AsA+DHA)	DHA/AsA ratio
Genotypes (A)					
'Ailsa Craig'	1.59 b[a]	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	<0.0001	<0.0001	<0.0001	0.612	0.643
Duration (B)					
0 h	2.20	3.09	5.29	0.41	1.58
2.5 h	2.07	3.29	5.36	0.36	1.82
5 h	1.53	2.64	4.17	0.33	2.14
P-value, linear	<0.0001	0.066	0.001	0.004	<.0001
P-value, non-linear	0.140	0.029	0.030	0.451	0.568
(A)×(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)×(C) *P*-value	0.903	0.956	0.755	0.934	0.789
(B)×(C) *P*-value	0.583	0.228	0.481	0.119	0.055
(A)×(B)×(C) *P*-value	0.990	0.881	0.979	0.953	0.481

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

Table 6. *Reduced ascorbic acid (AsA), dehydroascorbic acid (DHA), total AsA+DHA, reduced glutathione (GSH), glutathione disulphide (GSSG), and total GSH+GSSG concentrations, redox ratios [AsA/(AsA+DHA) and GSH/(GSH+GSSG)] and DHA/AsA and GSSG/GSH ratios in exocarp from exposed and covered sections of immature green fruit after 5 h of exposure*

P-value indicated for comparison within rows.

Variable	Exposed	Covered	*P*-value
AsA (μmol g^{-1} FW)	1.52	1.89	0.057
DHA (μmol g^{-1} FW)	2.60	3.08	0.072
AsA+DHA (μmol g^{-1} FW)	4.12	4.98	0.026
Redox ratio(AsA/AsA+DHA)	0.34	0.37	0.298
DHA/AsA ratio	2.27	2.08	0.616
GSH (μmol g^{-1} FW)	0.138	0.154	0.091
GSSG (μmol g^{-1} FW)	0.004	0.006	0.214
GSH+GSSG (μmol g^{-1} FW)	0.142	0.160	0.135
Redox ratio (GSH/GSH+GSSG)	0.930	0.990	0.106
GSSG/GSH ratio	0.089	0.069	0.639

a lower *R-/S-* ratio (Table 11). 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 (data not shown).

Discussion

Surface bleaching and discoloration are common symptoms of photo-oxidative damage of tomato fruit (Ramsey *et al.*, 1952; Tomes *et al.*, 1956; Retig and Kedar, 1967; Rabinowitch *et al.*, 1974). In this study, it was found that the method of inducing photo-oxidative damage on detached and susceptible immature-green tomato fruit under natural conditions was effective in accelerating the development of typical sunscald symptoms that are seen visually on attached fruit exposed to full sunlight. Both high temperatures and solar irradiance caused fruit surface discoloration (Table 2), but it was solar irradiance that was most responsible for the typical bleaching symptoms in the immature green exocarp of these tomato genotypes, which increased with duration of exposure to solar irradiance (Table 1).

Fruit discoloration from green towards a yellow colour, caused by high temperature either with or without direct sunlight, was related to a decrease of Chl relative to Car concentrations (Table 2). This apparent greater photostability by carotenoids has been previously reported in apple fruit by Merzlyak and Solovchenko (2002). Furthermore, Merzlyak *et al*. (2002) reported that sunscald-tolerant apple cultivars (e.g. 'Zhigulevskoye') build up large amounts of carotenoids in their sunlight-exposed fruit peel as a response to higher irradiance. They hypothesized that Car serve as photo-protectants in these cultivars. These authors also indicated that this increase in carotenoids does not occur in sunscald-susceptible cultivars (e.g. 'Granny Smith'), where both carotenoids and chlorophyll decline in sunlight-exposed fruit peel. Car and Chl also declined in the exocarp of immature green tomato fruit in our study.

Table 7. *Reduced (GSH), oxidized (GSSG), and total glutathione (GSH+GSSG) concentrations, and the redox [GSH/(GSH+GSSG)] and GSSG/GSH ratios in exocarp 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 Bonferroni adjustment.

Factors	GSH (nmol g^{-1} FW)	GSSG (nmol g^{-1} FW)	GSH+GSSG (nmol g^{-1} FW)	Redox ratio (GSH/GSH+GSSG)	GSSG/GSH ratio
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 (B)					
0 h	234	5.1	239	0.98	0.025
2.5 h	207	5.0	212	0.98	0.025
5 h	138	4.2	142	0.94	0.088
P-value, linear	<0.0001	0.840	<0.0001	0.782	0.082
P-value, non-linear	0.140	0.920	0.142	0.654	0.420
(A)×(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)×(C) *P*-value	0.499	0.859	0.512	0.678	0.730
(B)×(C) *P*-value	0.601	0.707	0.585	0.837	0.429
(A)×(B)×(C) *P*-value	0.772	0.936	0.732	0.654	0.855

Table 8. *Antioxidant enzyme (SOD, APX, DHAR, MDHAR, GR, CAT) activities expressed on a fresh weight (FW) basis from exocarp 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 Bonferroni 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[a]	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 (B)						
0 h	168	13.7	2110	6.7	2.80	1.53
2.5 h	183	13.9	1804	6.5	1.13	0.65
5 h	172.0	10.9	1724	8.2	0.61	0.66
P-value, linear	1.120	0.003	0.056	0.209	<0.0001	<0.0001
P-value, non-linear	0.017	0.082	0.950	0.300	0.001	0.006
(A)×(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)×(C) *P*-value	0.114	0.319	0.729	0.743	0.470	0.085
(B)×(C) *P*-value	0.876	0.478	0.656	0.293	0.588	0.888
(A)×(B)×(C) *P*-value	0.365	0.332	0.587	0.880	0.861	0.598

[a] Different letters within columns indicate statistical differences. Tukey HSD ($P < 0.05$).

Interestingly, chlorophyll degradation and an increase in Car/Chl ratio are also part of ripening and senescence processes in many fleshy fruits, including tomatoes (Torres, 2001; Andrews *et al*., 2004). Andrews *et al*. (2004) reported that as tomatoes ripened the Chl *a*/Chl *b* ratio in exocarp declined linearly, which is opposite to this study's results with immature green fruit during the development of photo-oxidative damage (Table 3). Similarly, an increase in Chl *a*/Chl *b* ratio was reported when shaded leaves were transferred to full sunlight (Burritt and 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 photo-oxidative damage or sunscald.

Hp-1 (*hpA* and *hpR*) fruit exocarp had significantly higher Chl and Car contents than the other genotypes (Table 3), as has been previously reported for both exocarp and mesocarp tissues (Thompson, 1955, 1961; Baker and Tomes, 1964; Clayberg *et al*., 1970; Jarret *et al*., 1984; Stevens and Rick, 1986; Torres, 2001; Andrews *et al*., 2004). This characteristic of *hp-1* fruit was directly related to differences in surface colour parameters. Hue angle, the parameter that represents colour, was similar in all genotypes, including the two *hp-1* mutants (Table 1). However, the green coloration of the *hp-1* mutants was more saturated (lower C) and of a darker (lower L*) green colour than the other genotypes (Table 1). Thus, the *hp-1* mutants, especially *hpA*, appeared most visually tolerant to the imposed photo-oxidative stress, in agreement with previous findings (Torres, 2001). The lower values of L* and C probably resulted from the higher Chl and Car concentrations in *hp-1* exocarp (Table 3). This apparent tolerance, however, was not detectable by the method of measuring surface colour that was employed, since there was no significant interaction between genotypes and

Table 9. *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 after 5 h of exposure*

P-value indicated for comparison within rows.

Variable	Exposed	Covered	*P*-value
SOD (units $min^{-1}g^{-1}$ FW)	173	162	0.444
APX (μmol AsA min^{-1} g^{-1} FW)	10.8	12.9	0.000
DHAR (nmol AsA min^{-1} g^{-1} FW)	1707	1572	0.287
MDHAR (μmol NADH min^{-1} g^{-1} FW)	8.3	6.3	0.097
GR (μmol NADPH min^{-1} g^{-1} FW)	0.63	1.36	<0.0001
CAT (AU min^{-1} g^{-1} FW)	0.66	1.11	0.031
Protein (mg g^{-1} FW)	1.8	2.7	0.003
SOD (units $min^{-1}g^{-1}$ protein)	711	186	0.001
APX (μmol AsA $min^{-1}g^{-1}$ protein)	30.3	10.6	0.002
DHAR (nmol AsA min^{-1} g^{-1} protein)	1459	897	0.001
MDHAR (μmol NADH min^{-1} g^{-1} protein)	3199	553	0.002
GR (μmol NADPH min^{-1} g^{-1} protein)	97.3	65.7	0.236
CAT (AU min^{-1} g^{-1} protein)	2.37	0.20	0.445

Table 10. *Total protein and antioxidant enzymes (SOD, APX, DHAR, MDHAR, GR, CAT) activities expressed on a protein basis in exocarp 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 Bonferroni adjustment.

Factors	Protein (mg g^{-1} FW)	SOD (units min^{-1} g^{-1} protein)	APX (μmol AsA min^{-1} g^{-1} protein)	DHAR (nmol AsA min^{-1} g^{-1} protein)	MDHAR (nmol NADH min^{-1} g^{-1} protein)	GR (nmol NADPH min^{-1} g^{-1} protein)	CAT (AU min^{-1} g^{-1} protein)
Genotypes (A)							
'Ailsa Craig'	3.01 a[a]	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	<0.0001	0.139	0.014	0.303	0.120	0.406	0.049
Duration (B)							
0 h	3.74	56.9	4.02	623	202	84.1	0.11
2.5 h	2.89	87.2	6.06	941	333	44.4	0.08
5 h	1.91	1064	29.6	1438	3113	94.5	2.34
P-value, linear	<0.0001	<0.0001	<0.0001	0.001	<0.0001	0.002	0.284
P-value, non-linear	0.950	0.126	0.322	0.948	0.070	0.220	<0.0001
(A)×(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)×(C) *P*-value	0.415	0.525	0.271	0.527	0.839	0.115	0.183
(B)×(C) *P*-value	0.926	0.092	0.130	0.217	0.067	0.075	0.604
(A)×(B)×(C) *P*-value	0.055	0.426	0.372	0.242	0.263	0.617	0.414

[a] Different letters within columns indicate statistical differences. Tukey HSD (P <0.05).

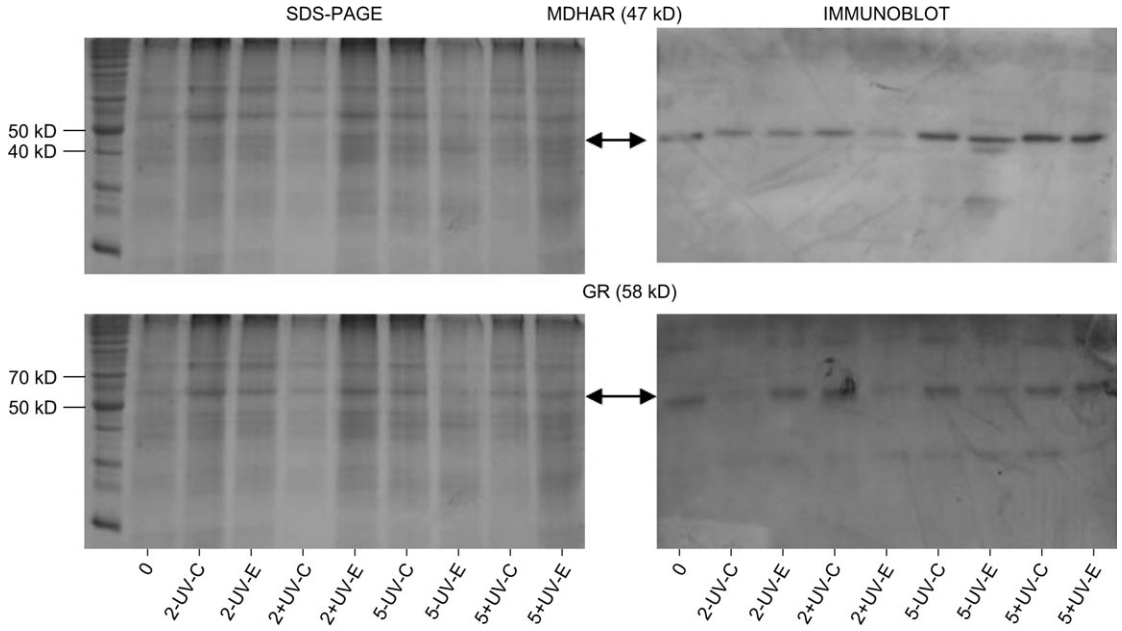

Fig. 3. SDS-PAGE and immunoblots of MDHAR and GR from immature green *hp-A* tomato exocarp (20 μg protein lane^{-1}) from photo-oxidative stress treatments indicated by duration of exposure (0, 2, or 5 h), presence or absence of UV radiation (+UV or −UV), and covered (C) or exposed (E) fruit sections. MDHAR (47 kDa) cross-reacted with anti-*Zea mays* MDHAR and GR (58 kDa) cross-reacted with anti-spinach GR.

Table 11. *Total kaempferol (K), quercetin (Q),* R-*naringenin,* S-*naringenin, and total naringenin concentrations and Q/K and* R-*naringenin/*S-*naringenin* (R-/S-) *ratios from exocarp of immature green fruit from 'Ailsa Craig' and hpA on sections of exposed fruit*

P-values indicated for comparisons within columns. Trend contrasts *P*-values represent a Bonferroni adjustment.

Factors	Kaempferol (μg g^{-1} FW)	Quercetin (μg g^{-1} FW)	Q/K	*R*-naringenin (μg g^{-1} FW)	*S*-naringenin (μg g^{-1} FW)	Total naringenin (μg g^{-1} FW)	*R*-/*S*- ratio
Genotypes							
'Ailsa Craig'	185.7	831.9	5.2	53.4	59.8	113.2	0.91
hpA	277.7	4280.2	14.9	75.0	140.1	215.1	0.56
P-value	0.042	0.033	0.059	0.056	0.010	0.010	0.001

duration of exposure for L*, C, or hue angle during the 5 h of photo-oxidative stress (Table 1).

Fruit exocarp of the *hp-1* mutants did not show any tolerance to photoinhibition, measured as F_v/F_m (Table 4). This suggests that their visually higher tolerance to photo-oxidative stress may not be due to protection of the reaction centres by their additional carotenoids, but to other antioxidant components, such as ascorbate and some ascorbate–glutathione cycle enzymes, which are enhanced in these mutants (Jarret *et al*., 1984; Torres, 2001; Andrews *et al*., 2004).

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 a reduction in the F_v/F_m ratio, in the first 2.5 h of exposure. The presence of sunlight accounted for the remaining 22% decrease of an overall 75% decrease in F_v/F_m. 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 dramatically in these species. This temperature threshold ranged between 42–47 °C in high-temperature-adapted species (Seemann *et al*., 1984). Ludlow and Björkman (1984) also described a similar phenomenon in *Macroptilium atropurpureum* 'Siratro' leaves at temperatures over 42 °C under high irradiance, which they called 'high-temperature-induced photoinhibition'. In our study, thermoinhibition was detected after 2.5 h of exposure when fruit surface temperatures averaged 46 °C. It is also possible that this high-temperature effect occurred prior to 2.5 h of exposure at lower surface temperatures, especially since Smillie *et al*. (1999) showed that PSII efficiency decreased more rapidly in fruit than in leaves with increasing PPFD. Thermoinhibition, as well as photoinhibition, could cause increases in cellular ROS flux and possible up-regulation of antioxidant systems to cope with the increased ROS.

The results suggest that antioxidant metabolites and enzymes could have been exerting some degree of protection from photo-oxidative damage under these experimental

conditions. This is supported by the increased activities of antioxidant enzymes as duration of exposure increased (Table 10). If the photo-oxidative stress conditions had persisted, however, the antioxidant systems may have been overcome at some point, leading to permanent photo-oxidative injury, especially in non-aclimated fruit tissue.

In agreement with Adegoroye and Jollife (1987) and Prohens *et al*. (2004), AsA levels decreased in fruit exocarp as duration of exposure increased (Table 5). Similar to these results, a decline in ascorbate content as a response to imposed oxidative stress in leaves has been reported in other studies (Wise and Naylor, 1987; Sairam *et al*., 1998). On the other hand, plant acclimation and/or plant tolerance to oxidative/photo-oxidative stress have resulted in higher ascorbate contents in both leaves and fruits (Gatzek *et al*., 2002; Logan *et al*., 1998; Ma and Cheng, 2003).

Although fruit ripening has been associated with oxidative stress, trends vary in AsA levels in different and even among similar species (Andrews *et al*., 2004; Jiménez *et al*., 2002; Lentheric *et al*., 1999; Torres, 2001). Unlike the effects of UV-B radiation on leaves (Hideg *et al*., 1997), no effect on the ascorbate pool by the presence or absence of natural UV radiation was seen (Table 5). The results also suggest that both intense visible sunlight and elevated temperatures were equally responsible for the decline in reduced AsA and DHA (Table 6). Similar to the findings of Foyer *et al*. (1989) for leaves, there were no differences in the redox state of ascorbate between covered and sunlight-exposed fruit (Table 6).

As found in this study and also reported by Andrews *et al*. (2004), the ascorbate pool was elevated in the exocarp of the *hp*-1 mutant (Table 5). Since ascorbate decreased across genotypes as photo-oxidative stress progressed, the higher ascorbate concentration in the exocarp of *hp-1* might confer this mutant with greater tolerance to photo-oxidative stress than the other genotypes.

Although glutathione has been used as an oxidative stress indicator in plants (Grill *et al*., 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 and Knowles, 1996;, Sgherri and Navari, 1995), others have found no increase in GSH by environmental stresses (Tausz *et al*., 2004). No increase was found in either GSH or GSSG as photo-oxidative stress progressed (Table 7). 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 the fruits had only reached an initial state of acclimation to photo-oxidative stress. This may in fact be the case, since the fruits were harvested from shaded positions within the plant canopy. 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 the findings of increased GSH levels in plants submitted to long-term environmental stresses (Polle and Rennenberg, 1992)

These data suggest that increasing GR activity (Table 10) was able to maintain the glutathione redox state [GSH/(GSH+GSSG)] at over 0.94 during exposure of the fruit to photo-oxidative stress (Table 7), thereby reducing GSSG to GSH via a NADPH-dependent reaction (Carlberg and Mannervik, 1985).

Despite the decline in total protein during exposure to solar radiation, the activities of all antioxidant enzymes on a protein basis dramatically increased as photo-oxidative stress progressed (Table 10). These results signify that the activities of these enzymes were conserved, perhaps because they were not targeted by proteinases under the 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 photo-oxidative stress progressed (Fig. 3), indicating that these enzymes were conserved under the stress conditions used here. This may represent an acclimation response to photo-oxidative stress. Indeed, MDHAR mRNA was found to increase in response to oxidative stresses generated by ozone, hydrogen peroxide, and methyl-viologen (Yoon *et al*., 2004). Similarly, increases in mRNA have been observed for other antioxidant enzymes, such as APX (Park *et al*., 2004) and DHAR (Urano *et al*., 2000), during oxidative stress.

UV radiation (+UV) increased APX and MDHAR activities on a protein basis (Table 10), and CAT activity on a FW basis (Table 8). Similarly, enhanced peroxidase (including APX) activities in *Arabidopsis thaliana* leaves exposed to UV-B irradiation were reported by Rao *et al*. (1996). In leaves, UV irradiation mainly stimulated the production of $O_2^{\cdot-}$, instead of $^1O_2$, whereas mostly $^1O_2$ production was stimulated when leaves were exposed to strong visible light (Hideg *et al*., 2002).

Drought and chilling stress, as well as other stresses, have been associated with oxidative stress, but for both drought and chilling stress different responses by antioxidant components have been reported (Gechev *et al*., 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 tomato fruit (Table 9). This indicates that high temperatures, even without the stress imposed by visible light, produced a similar response in these enzymes to an increase in ROS flux.

The *hp-1* mutants, particularly *hpA*, as well as having more elevated ascorbate pools in exocarp tissue than the other genotypes (Table 5), also had higher APX and GR activities (Table 8). 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 a reduction of oxidized glutathione (GSSH) in order to recycle DHA to AsA.

There were no detectable changes in concentrations of quercetin, kaempferol, or naringenin enantiomers in fruit exocarp as the duration of exposure increased (data not shown), yet kaempferol and especially quercetin accumulated rapidly when fruits were exposed to sunlight (Table 2). These results suggest that kaempferol and quercetin synthesis might be triggered as a photoprotective mechanism by a sudden increase in light levels as suggested by Close and 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 either the presence or absence of sunlight (data not shown).

The presence or absence of natural UV radiation did not have an effect on flavonoid accumulation (data not shown), as has been shown for leaves (Ryan *et al*., 2002). This suggests that the accumulation of flavonoids as a photoprotective mechanism might be rapidly engaged by a sudden increase of visible light, but not UV radiation. It is also possible that UV-B induction of flavonoids (Ryan *et al*., 2002; Solovchenko and 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 light (Jordan, 1996), minimizing photo-damage suffered by the photosynthetic apparatus under high light.

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 2). Curiously, although quercetin and kaempferol did not increase in the +UV treatment, the Q/K ratio did, from 7.9 for −UV to 10.6 for +UV (P=0.07). An increase in Q/K ratio might be a photoprotective mechanism that occurs with higher levels of UV-B radiation (Ryan *et al*., 2002). 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 *et al*., 1995), and so quercetin synthesis is favoured over kaempferol in light-stress conditions. However, an increasing Q/K ratio could be merely due to a faster rate of degradation of kaempferol than quercetin.

Previous reports on *hp-1* mutants have indicated the enhancement of anthocyanin levels in several plant parts (Wettstein-Knowles, 1968*a*; Jarret *et al*., 1984). In this study, each of the flavonoids analysed was greatly enhanced in *hpA* fruit exocarp compared with its parent, AC (Table 11). This is another pleitropic effect of the mutation on the HP genes, which are believed to be negative regulators of phytochrome signal translation (Kerr, 1965; Kerckhoffs *et al*., 1997; Peters *et al*., 1998; Mustilli *et al*., 1999).

In conclusion, in this study, the progression of photo-oxidative stress was followed in detached fruit under natural solar irradiance. Using this system, factors that were involved in the development of visual symptoms of sunscald and damage to the photosynthetic apparatus during photo-oxidative stress episodes were elucidated. These results suggest that antioxidant metabolites and enzymes may have been exerting some degree of protection to photo-oxidative damage. If the photo-oxidative stress conditions had persisted longer, however, the antioxidant systems may have been overcome, leading to permanent photo-oxidative injury. It was also determined that, as well as in leaves, flavonoids are rapidly induced in fruit exocarp exposed to solar irradiance as a physiological acclimatory response. It did not appear, however, that the natural UV radiation levels at the experimental site were responsible for either the increased flavonoids in the exocarp or sunscald development in any of the genotypes studied. Finally, the results suggest that during the photo-oxidative stress there was an increase in ROS flux due to decreased intrinsic PSII efficiency in the chlorophyll-containing exocarp of these immature tomato fruit. Although antioxidant metabolite pools initially decreased in response to photo-oxidative stress, the specific activities of antioxidant enzymes increased in order to maintain the ascorbate and glutathione pools in their reduced forms to eliminate ROS.

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