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Response of the Fruit Antioxidant System to the Post-Chilling Period in Two Different Tomato Lines

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ABSTRACT

Antioxidant systems play an important role in prevention of chilling injury, and chilling-tolerant fruit may initially contain more antioxidants or be able to maintain their antioxidant levels during stress. Tomato fruit are chilling sensitive and chilling symptoms generally appear after fruit are transferred to non chilling temperatures. The aim of this study was to characterise the events occurring at 20°C, immediately after a storage period at 4°C, and to establish the importance of tomato fruit ascorbic acid levels in the response to chilling. Two different tomato lines were used: a processing cultivar (M82) with low to average ascorbic acid levels and an introgression line (IL9.2.5) containing elevated ascorbic acid levels due to the introgression of wild *Solanum pennellii* alleles on chromosome 9. We show a rapid response (0.5 hours) of the antioxidant system in tomato to re-exposure to normal temperatures and light following chilling. The ascorbate redox state undergoes rapid changes and is followed by changes in the redox state of the glutathione pool. The response is different in the two tomato lines studied: fruits from IL9.2.5 have a higher ascorbic acid and glutathione redox state compared to the parent M82 where the pool of both antioxidants becomes oxidised. The redox state of the ascorbate pool is correlated with the activity of enzymes involved in ascorbate regeneration (monodehydroascorbate reductase and dehydroascorbate reductase) in the fruit. Furthermore the ability of IL9.2.5 to maintain reduced antioxidant levels is correlated with a decreased loss of fruit firmness when compared to M82.

Keywords: ascorbate, fruit firmness, glutathione, redox, *Solanum lycopersicum*, texture
Abbreviations: APX, ascorbate peroxidase; **DHAR**, dehydroascorbate reductase; **DTT**, dithiothreitol; **GR**, glutathione reductase; **GSH**, glutathione; **GSSG**, glutathione disulphide; **HPLC**, high pressure liquid chromatography; **MDHAR**, monodehydroascorbate reductase; **NADH**, nicotinamide adenine dehydrogenase; **PME**, pectin methyl esterase; **QTL**, quantitative trait locus; **TCA**, trichloroacetic acid

INTRODUCTION

Low temperature storage is often used to lengthen fruit shelf life, allowing an extended market period, long distance transport, and a more regulated supply of commodities to the consumer. However, storage of fleshy fruits (such as tomato) below 10°C triggers many physiological disorders, including loss of firmness, pitting, water-soaking, loss of flavour, development of off-flavours and failure to ripen (Lyons 1973; Hodges *et al*. 2004). This alteration of fruit quality induced by cold storage is called chilling injury: symptoms generally appear once fruits are allowed to ripen normally following a period of cold storage and lead to consumer dissatisfaction and reduced fruit consumption (Saltveit and Morris 1990). Because storage at low temperature remains a good way to protect harvested fruits from pathogens and to extend fruit shelf life, many different pre- and post-harvest strategies have been set up to reduce the negative effects of chilling, including variation of fertilization and irrigation regimes, position of fruit within the canopy, crop load and size, warming interruptions during cold storage, treatment with plant growth regulators and controlled atmosphere storage (Lurie and Crisosto (2005) for review). These strategies have led to short term solutions to chilling injury problems, however improving knowledge concerning the genetic basis of chilling sensitivity might permit selection of chilling resistant cultivars, and therefore provide long term solutions.

Many symptoms related to chilling injury have been described at the cellular level. Modifications of membrane lipids leading to membrane leakiness in response to low temperatures were proposed to be an initial molecular event (Lyons 1973; Nishida and Murata 1996). However, other observations have indicated that membrane modification is an indirect rather than direct symptom of chilling injury (Sharom *et al*. 1994). Modification of fruit texture in response to chilling has also been attributed to an abnormal solubilisation of cell wall pectin resulting from an imbalance in the activities of polygalacturonase and pectin methyl esterase (PME) which is caused by prolonged exposure to low temperature (Dawson *et al*. 1992; Lurie *et al*. 1994; Zhou *et al*. 1999; Brummell *et al*. 2004). Recently, evidence has accumulated that chilling injury is linked to oxidative stress. It has been demonstrated that profiles of antioxidant compounds were modified in response to low temperature, resulting in an alteration in the equilibrium between active oxygen species generation and defence mechanisms (Sala 1998; Toivonen and Sweeney 1998; Hodges *et al*. 2001). Hence, it has been postulated that antioxidant systems, which are able to counteract oxidative stress, may play an important role in prevention of chilling injury, and that chilling-tolerant plants or plant organs may initially contain more antioxidants or may be able to maintain or increase their antioxidant levels during stress (Wismer 2003).

Ascorbic acid (vitamin C) is an effective water-soluble antioxidant found in high concentrations in every compartment of plant cells. It can react with a wide range of active oxygen species and is involved in the ascorbate-glutathione cycle to detoxify hydrogen peroxide via the activity of four enzymes: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor 2006).

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Improvement of ascorbic acid content in species of agronomic interest is cited as an important criterion because as well as having well-known nutritional benefits for the consumer (Agius *et al*. 2003; Davuluri *et al*. 2005; Hancock and Viola 2005; Paine *et al*. 2005), ascorbic acid content can contribute to both biotic and abiotic stress tolerance in crops (Davey *et al*. 2000; Muckenschnabel *et al*. 2002; Kuzniak and Sklodowska 2005). Furthermore, ascorbic acid content seems to be involved in post-harvest fruit quality as ascorbic acid levels have been linked to flesh browning in pear and these levels, rather than total fruit antioxidant activity, appear to be linked to improvement of shelf life in apple (Veltman *et al*. 1999; Davey *et al*. 2006). This provides an additional reason to focus on enhancement of ascorbic acid content in agronomic crops.

Fruit ascorbic acid content is highly influenced by the environment but also by genetically determined factors, such as the level of synthesis, recycling, degradation and transport of this molecule within the cell or between organs. A quantitative trait locus (QTL) controlling ascorbic acid content has been identified on tomato chromosome 9; the introgression of alleles from the wild species *Solanum pennellii* into the genetic background of M82 (a processing cultivar) increases the fruit ascorbic acid content by up to 100% when compared with the *Solanum lycopersicum* alleles from M82 (Stevens *et al*. 2007). The introgression line containing alleles from the wild species is called IL9.2.5. An MDHAR gene colocates with this QTL. MDHAR is a reductase that allows regeneration of the reduced form of ascorbic acid from its unstable oxidized radical, monodehydroascorbate, avoiding depletion of the ascorbate pool: if not rapidly rereduced to ascorbate, oxidised ascorbate is catabolised via dehydroascorbate to two- and four- carbon products such as oxalate and tartrate (Noctor and Foyer 1998; Smirnoff and Wheeler 2000). The role of the MDHAR enzyme in stress responses has been demonstrated for many kinds of oxidative stress (Grantz *et al*. 1995; Yoon *et al*. 2004). It has also been shown that under chilling conditions in tomato, MDHAR activity explained 84% of reduced ascorbic acid levels in fruit compared with 38% at harvest under non stress conditions. Furthermore, increased fruit MDHAR activity, and a lower oxidation level of the fruit ascorbate pool, has been correlated with decreased loss of firmness caused by chilling injury (Stevens *et al*. 2008). These results indicate a role for ascorbic acid and its recycling pathway in fruit tolerance to cold storage conditions but do not exclude a role for other antioxidants or ascorbate recycling enzymes.

As chilling symptoms generally appear after tomatoes are transferred to non chilling temperatures (Saltveit and Morris 1990), when re-exposure to normal temperatures and light occurs, the aim of this study was to characterise the events occurring at 20°C, immediately after a storage period at 4°C, and to establish the importance of ascorbic acid levels in this response. To achieve this, the two different tomato lines described above (M82 and IL.9.2.5) were used. Our previous results showed that the introgression line is more tolerant to chilling but the events that occur rapidly post-chilling have not been characterised (Stevens *et al*. 2008). Therefore in this study, levels of ascorbic acid, glutathione and activities of the enzymes involved in the ascorbate-glutathione cycle were measured immediately after chilling and during the warming period that followed. These were compared to fruit firmness measurements, fruit weight loss and fruit methanol content (as an indictor of pectin methyl esterase activity) in order to identify the origin of differences observed in fruit firmness. The results give an indication of the importance of the redox state of the ascorbate and glutathione pools and the rapid response of the ascorbate pool following chilling stress.

MATERIALS AND METHODS

Growth and cold storage conditions and sampling of tomato lines

The introgression line IL9.2.5 originated from a population of introgression lines consisting of 75 lines each containing a single introgression fragment from the wild tomato accession *Solanum pennellii* LA716 (*S. pennellii*) in the genetic background of M82, a processing tomato variety with determinate growth (Eshed and Zamir 1995). The M82 parent and the IL9.2.5 were grown in the greenhouse in Avignon (South East France) in Spring 2006 and for each genotype a minimum of 6 fruits per time point per experiment were harvested at breaker or orange stages from 10 plants on 19th May 2006 (experiment 1) and 24th May 2006 (experiment 2). The harvested fruits were stored in the dark at 4°C for 26 days. At the end of the chilling period fruits were transferred to room temperature (20°C) in the light. Fruits were weighed and firmness measurements were carried out on fruits at harvest and then 0, 0.5, 1.5, 4, 19 and 27 hrs following the end of the chilling period (a minimum of 6 fruits per time point). Following these measurements, transversal sections were cut from the middle of each fruit, (including pericarp, columella, and the jelly-like locular tissues), frozen and ground to a fine powder in liquid nitrogen and stored at -80°C for the biochemical assays described below.

Measurement of tomato weight and firmness

Firmness is an overall estimation of fruit resistance to compression and is a combination of skin resistance and flesh firmness (Grotte *et al*. 2001). Fruit firmness was assessed with a texturometer (Texture analyser TAplus: Ametek, Lloyd Instruments Ltd., Fareham, UK). This apparatus registered force/deformation curves by measuring the reaction force in response to an increasing mechanical constraint applied to the fruit by a 5 cm flat disc supported by a motorised arm. Fruit firmness was equivalent to the force necessary to obtain a deformation corresponding to 3% of the fruit diameter. The probe speed was 20 mm min^{-1}. Firmness was measured at harvest, and then at each time point of the trial (warming period). Loss of firmness (expressed as a percentage of the initial harvest value) corresponds to the relative differences observed. At the same time, fruits were weighed and fruit weight loss was calculated in the same way. Fruit weight loss is an indirect measurement of the loss in water content of the fruit.

Methanol content of fruit tissues

Methanol content in fruit tissues results from the removal of methyl residues from pectins. The measurement was performed directly, without previous saponification, and therefore most modifications of the methanol content should result from the methylesterification of pectins through the action of pectin methyl esterase (PME) (Koch and Nevins 1989). Methanol measurement was therefore an indirect method for estimating the PME activity. Methanol was measured as previously described (Renard and Ginies 2009) with a few modifications. Briefly, methanol content was determined in samples by Headspace-GC–MS. Samples of 2 mg of powder were added to 2 ml of phosphate buffer (pH 2) containing CD_3OH (7 µmol.ml^{-1}) as an internal standard. A calibration curve was prepared with methanol in the range 0.6–6 µmol ml^{-1} with CD_3OH as an internal standard as above. The GC–MS QP2010 Shimadzu with capillary column Cp_wax_52cb 30 m × 0.32 mm × 0.5 µm (Varian, Inc, Palo Alto, USA) equipped with auto sampler AOC5000 was used for measurements. The sealed vial was placed at 50°C for 15 min and then 0.5 ml of head-space was injected into the split injector (ratio 1:10). GC conditions were: helium as gas carrier at 45 cm s^{-1}, oven temperature isothermal at 40°C. Mass detector conditions were: electronic impact ionisation mode: 70 eV, source temperature: 200°C with data collected using SIM for selected ions (m/z 31; 32; 35) at 5 scans/s. Methanol concentrations were calculated by plotting the peak area ratio (normal to deuterated forms) for ion pairs (m/z 31/35) over a range of concentration ratios (CH_3OH/CD_3OH). The ion at m/z 35 is the molecular ion for CD_3OH.

Ascorbic acid content

Measurements of ascorbic acid content were carried out as described by Stevens *et al*. (2006) on material conserved at -80°C. Briefly, 1 g of powder was homogenised with 600 µl of ice-cold 6% trichloroacetic acid (TCA). Samples were centrifuged for 15 min at 25000 × *g* at 4°C. Twenty microlitres of the supernatant were used in each assay. The ascorbate standards were prepared fresh: a solution of 1 mg/ml sodium ascorbate was diluted in 6% TCA to give a concentration in 20 µl of 0, 5, 10, 15, 20 and 30 nmoles allowing a standard curve of absorption values of between 0 and 1 to be generated after addition of the appropriate reagents (below). Two assays were carried out on each sample: one to measure the total ascorbate (including addition of 5 mM dithiothreitol (DTT)) and one to quantify the reduced ascorbate content (omission of DTT from the assay). Twenty microlitres of each sample or standard were distributed into wells of a 96-well microplate and mixed with 20 µl of 5 mM DTT (total ascorbate assay) or 0.4 M phosphate buffer pH7.4 (reduced ascorbate assay). The plate was incubated at 37°C for 20 min. Ten microlitres of N-ethyl malemide (total ascorbate assay) or 0.4 M phosphate buffer pH 7.4 (reduced ascorbate assay) were added and mixed followed by the addition of 80 µl of colour reagent (see below). After incubation at 37°C for 40 min, the absorbance was read at 550 nm using a microplate reader (Thermo Electron). The colour reagent was made up as follows: solution A: 31% orthophosphoric acid, 4.6% (w/v) TCA and 0.6% (w/v) iron chloride; solution B: 4% 2,2-dipyridyl (w/v made up in 70% ethanol). Solutions A and B were mixed 2.75 parts (A) to 1 part (B). The standard curve obtained from the standard solution values allowed calculation of the ascorbate concentration of the samples after correction for the quantity of water introduced by the tomato fruit sample. Extractions and assays were carried out in triplicate.

HPLC analysis of glutathione and glutathione disulphide

Glutathione (GSH) and glutathione disulphide (GSSG) were extracted on ice in 0.1 M HCl from about 30 mg of freeze-dried, finely-ground tomato material and separated from cysteine, cysteinyl-glycine and γ-glutamyl cysteine and from their disulphides by reversed-phase HPLC (Jasco) (Kranner and Grill 1996). Briefly, this assay uses fluorescence labelling of thiols by monobromobimane. Total thiol (reduced + oxidised) concentrations were determined after reduction of disulphides by dithiothreitol (DTT). For determination of disulphides, thiol groups were blocked with *N*-ethylmaleimide (NEM). Excess NEM was removed and the remaining disulphides were reduced with DTT and analysed as above. Analytical HPLC conditions were the following: the stationary phase was a HIQ sil C18V HPLC column (4.6 mm x 250 mm) (Kya tech). The mobile phase was a gradient composed of solvent A (0.25% (v/v) acetic acid, adjusted to pH 3.9 with 10 M NaOH and filtered through a membrane filter (0.45 µm pore size)) and of methanol (Solvent B). The gradient mode at a flow rate of 1 mL min-1 was applied during the run. The gradient composition was the following: Solvent B ramped from 12 to 27% during the first 15 min then it attained 100% in the following 4 min, stayed constant for a further 7 min, and decreased down to 12% within the last 0.5 min of the run. Thiols were detected using a fluorescence detector. The excitation and emission wavelengths were set at 380 and 480 nm, respectively. This HPLC technique allowed, in the following order of elution, the separation of: cysteine, cysteinyl-glycine, γ-glutamyl-cysteine, glutathione and DTT.

Monodehydroascorbate reductase activity

Assay of MDHAR activity was carried out according to a previously described method (Arrigoni *et al*. 1981) based on the oxidation of NADH at 30°C measured at 340 nm. Approximately 150 mg of frozen powder was homogenised with 600 µl of extraction buffer (50 mM Tris-HCl pH 7.8). After centrifuging at 25,000 × *g*, 4°C for 15 min, 50 µl of the clear supernatant was assayed in a final volume of 1 ml containing 1 mM ascorbate, 0.2 mM NADH and enough ascorbate oxidase enzyme to give a linear rate of production of the monodehydroascorbate radical as measured at 260 nm (approximately 0.1 U). The initial rate without the ascorbate oxidase enzyme was subtracted from the final rate once this enzyme was added to the reaction. Results were expressed per µg protein. Extractions and assays were carried out in triplicate.

Dehydroascorbate reductase activity

DHAR activity was assayed mainly as previously described (Dalton *et al*. 1986). Tomato fruit were ground in liquid nitrogen and the soluble protein was obtained by centrifuging (25,000 × *g*) a tube containing approximately 150 mg of this powder in 1 mL of 50 mM Hepes-NaOH buffer pH = 7.5. Fifty microlitres of the clear supernatant were put in a final volume of 1 mL containing 50 mM of potassium phosphate pH 6.5, 1 mM of EDTA and 2 mM of DHA, and the absorbance was measured at 265 nm. The initial rate due to a non enzymatic reduction of DHA by 2.5 mM GSH without the extract was subtracted from the final rate once the extract was added to the reaction. Results were expressed per µg protein. Extractions and assays were carried out in triplicate.

Ascorbate peroxidase activity

Assay of total APX activity was carried out according to a previously described method (Nakano and Asada 1987) based on the oxidation of ascorbate at 30°C measured at 290 nm. Approximately 150 mg of frozen powder was homogenised with 600 µl of extraction buffer (50 mM potassium phosphate pH 7.0; 2 mM ascorbic acid). After centrifuging at 500 × *g*, 4°C for 10 min, 50 µl of the clear supernatant was assayed in a final volume of 1 ml containing 0.25 mM ascorbate and 5 mM H_2O_2. The initial rate in the absence of H_2O_2 was subtracted from the final rate once the reaction was started. Results were expressed per µg protein. Extractions and assays were carried out in triplicate.

Glutathione reductase activity

Assay of GR activity was carried out according to a previously described method (Edwards *et al*. 1990) based on the oxidation of NADPH at 30°C measured at 340 nm. Approximately 150 mg of frozen powder was homogenised with 600 µl of extraction buffer (50 mM HEPES pH 7.8, 0.5 mM EDTA). After centrifuging at 25,000 × *g*, 4°C for 10 min, 50 µl of the clear supernatant was assayed in a final volume of 1 ml containing 0.1 mM NADPH and 0.5 mM GSSG. The initial rate in the absence of GSSG was subtracted from the final rate once the reaction was started. Results were expressed per µg protein. Extractions and assays were carried out in triplicate.

Protein assays

Protein assays were carried out in triplicate using the method of Bradford (1976). Bovine serum albumin was used as a standard.

RESULTS

Changes in fruit firmness and weight following the end of chilling in M82 and IL9.2.5

The firmness and the weight of fruits were compared during the warming period to the measurements carried out at harvest, before the chilling period. Measurements were carried out on each fruit individually to allow a precise evaluation of the intra-sample variability (presented as boxplots in **Fig. 1**) and the results of the two independent experiments have been combined. M82 showed a higher loss of firmness at all time points compared to IL9.2.5 and this loss is highly significant ($p<0.001$) 1.5 and 4 hrs following the end of chilling whereas at 19 and 27 hrs post-chilling, the differences between the two lines were not significant (**Fig. 1A**). In contrast, the differences in weight loss between the two genotypes were not significant at any time point during the warming period (**Fig. 1B**). This result showed that the firmness lost is not a consequence of a sudden loss in fruit water content, but rather related to a transformation of the fruit tissues.

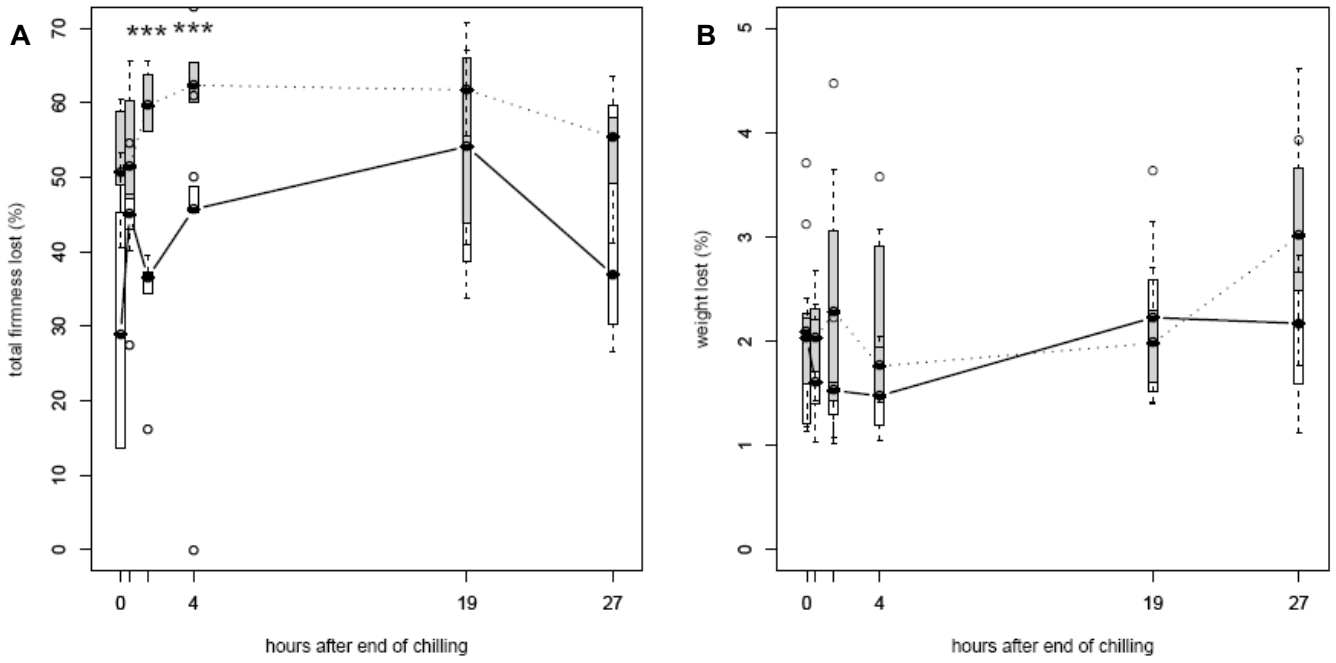

Fig. 1 Loss of fruit firmness and fruit weight compared to harvest for M82 (grey bars) and IL9.2.5 (white bars) at 0, 0.5, 1.5, 4, 19 and 27 hours after the end of chilling (26 days at 4°C). Bars show the 25-75% quartile range and the heavy circles the mean values and circles the extreme points. Stars indicate values that are significantly different between the two lines ($p<0.001$). The values are averages from the two independent experiments. (**A**) Total firmness lost (3% compression) (%). (**B**) Fruit weight loss (%).

Methanol content in fruit following the end of chilling

As previously observed for other chilled fruit (Brummell *et al*. 2004), pectin methyl esterase (PME) activity undergoes rapid changes and is therefore a marker for pectin modification leading to fruit softening during post-harvest. PME activity is estimated by measuring the methanol content in fruit tissues, without performing previous saponification. In ripening fruit, and under these conditions, most methanol measured in the tissues is a by-product of PME activity on pectin. At 0 hrs, the methanol content did not differ between the two genotypes (around 0.2 mg MeOH.gr^{-1}) (**Fig. 2**). Few differences were then observed during the trial, but none were significant when the two genotypes were compared. These results would tend to exclude a correlation between PME activity and the loss of firmness reported above.

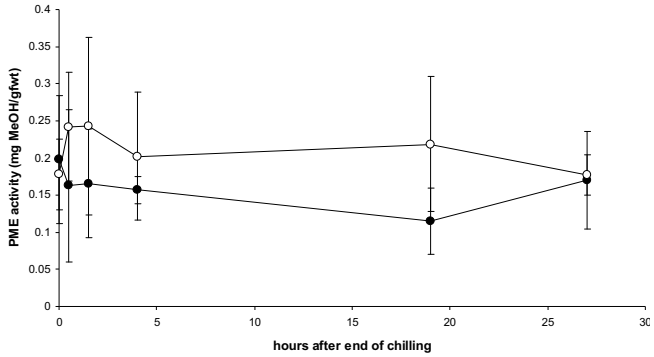

Fig. 2 Methanol content of M82 fruit (solid circles) and IL9.2.5 fruit (open circles) at 0, 0.5, 1.5, 4, 19 and 27 hours after the end of chilling (26 days at 4°C). Results are expressed in mg of methanol/g. The values are averages from the two independent experiments and are an indirect measure of pectin methyl esterase activity.

Changes in ascorbic acid and glutathione levels in M82 and IL9.2.5 during the warming period following chilling

The introgression line IL9.2.5, which carries a QTL enhancing its fruit ascorbic acid content, has been shown to be more resistant to chilling injury compared to the M82 parent line (Stevens *et al*. 2008). This advantage in terms of chilling tolerance has been correlated to the redox state of the ascorbate pool (defined as the ratio of the reduced form to the sum of the oxidised and reduced forms). As ascorbic acid and glutathione are major fruit antioxidants, their levels in the M82 and IL9.2.5 tomato lines were evaluated during the warming period after storage at 4°C. The evolution of total ascorbic acid levels of fruits is shown in **Fig. 3A**. Immediately following chilling, IL9.2.5 contains significantly more ascorbic acid than M82 (28 and 23 mg/100 gfwt respectively). After 0.5 hrs, M82 exhibited a slight decrease in ascorbic acid levels, followed by a gradual increase up to 4 hrs and then stabilisation reaching a final value of 26 mg/100 gfwt at 27 hrs post-chilling. In the case of IL9.2.5 fruits, the post-chilling period is followed by an increase in total ascorbic acid levels from 28 mg/100 gfwt at 0 hours to 34 mg/100 gfwt at 4 hrs followed by a gradual decrease to 30 mg/100 gfwt at 27 hrs post-chilling. The increase in IL9.2.5 compared to M82 was significant except at 27 hrs. The redox state of the ascorbate pool varied greatly between the two lines as shown in **Fig. 3B**. Although immediately following chilling the redox state of the fruit ascorbate pool of the two lines was not significantly different, after 0.5 hrs following chilling, the redox state of the M82 ascorbate pool decreased to 0.46 whereas for IL9.2.5 there was an increase to 0.94. The M82 ascorbate redox state then recovered by 4 hrs following chilling and stabilised at 0.67 whereas for IL9.2.5 the redox state decreased slightly by 4 hrs and stabilised at 0.89. The increased redox state of the ascorbate pool in IL9.2.5 at 0.5 hrs corresponds to an increase in the reduced form of ascorbate and a decrease in the oxidised form compared to M82.

Total glutathione levels for the two tomato lines are shown in **Fig. 3C**. Levels were similar in the two lines immediately following the end of chilling. In both lines, the

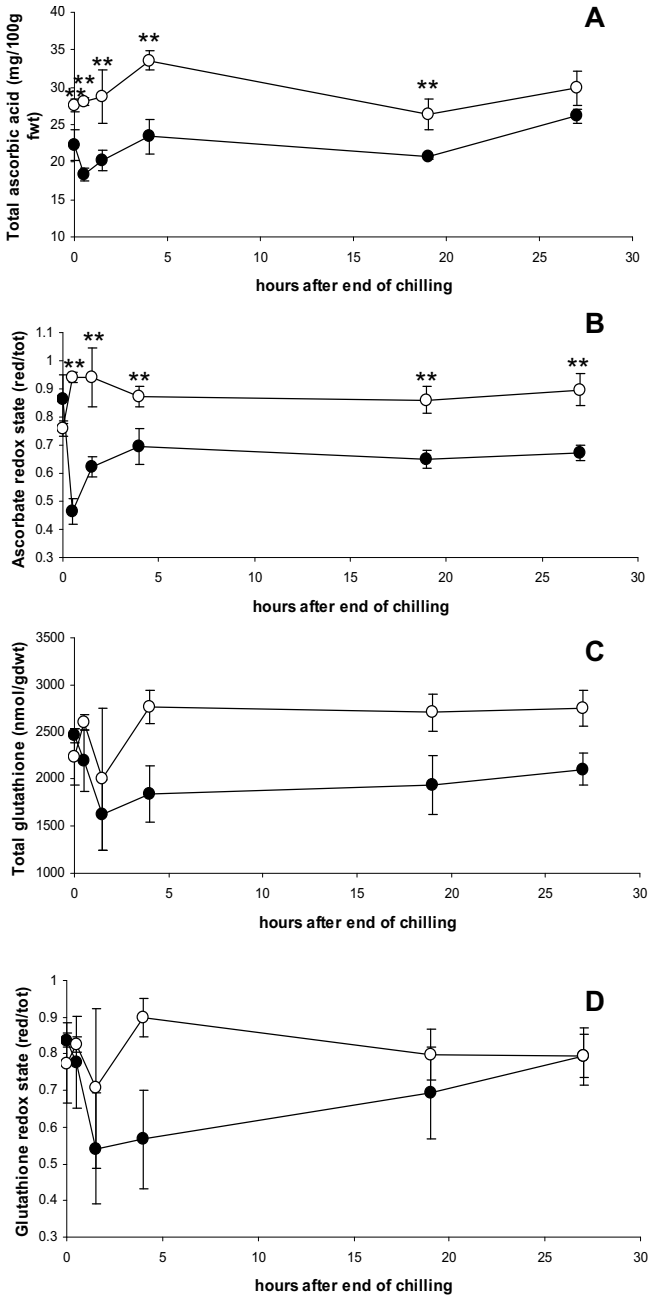

Fig. 3 Soluble antioxidant content in M82 (solid circles) and IL9.2.5 (open circles) during the post-chilling period at 0, 0.5, 1.5, 4, 19 and 27 hours following the end of the chilling period (26 days at 4°C). The values are averages from the two independent experiments. (**A**) Total ascorbic acid levels in fruit of M82 and IL9.2.5. Results are expressed in mg/100 gfwt. (**B**) Ascorbate redox state in fruit of M82 and IL9.2.5, defined as the ratio of reduced to total ascorbate. (**C**) Total glutathione levels in fruit of M82 and IL9.2.5. Results are expressed in nmol/gdwt. (**D**) Glutathione redox state in fruit of M82 and IL9.2.5, defined as the ratio of reduced glutathione (GSH) to 'total' glutathione (GSH+GSSG). Asterisks indicate significant differences (*: $p<0.05$; **: $p<0.01$).

end of chilling was followed by a decrease in total glutathione levels, and a minimum was reached at 1.5 hrs post-chilling. From 1.5 to 4 hrs post-chilling, an increase in these levels appears both in M82 and IL9.2.5, but this increase is greater in IL9.2.5. From 4 to 27 hrs post-chilling, IL9.2.5 had nearly a third more total glutathione compared to M82. In terms of the redox state of the glutathione pool (**Fig. 3D**), more marked differences are seen between the two lines as was the case for the ascorbate redox state. The end of cold storage of M82 tomato fruits is followed by a decrease in the redox state of the glutathione pool which corresponds to an increase in the oxidised form (GSSG). The redox state reaches a minimum of 0.54 at 1.5 hrs following the chilling period whereas for the line IL9.2.5 a maximum of 0.90 is reached at 4 hrs following the end of the chilling period. The reducing capacity of glutathione seemed to be high and to remain unaltered by cold storage in IL9.2.5 tomatoes as glutathione remained mainly under the GSH form (≥ 70%) following chilling. Compared to the changes seen in the ascorbate redox state which reached a maximum 0.5 hrs after the end of the chilling period, the changes in the glutathione redox state reach a maximum slightly later, at 1.5-4 hrs following the end of the chilling period. The redox state of the M82 glutathione pool returns to a level similar to that found for IL9.2.5 (0.79) after 27 hrs of warming.

Changes in the activity of enzymes involved in the ascorbate-glutathione cycle following the end of chilling

The activities of the four enzymes of the ascorbate-glutathione cycle: monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX) and glutathione reductase (GR) which use and regenerate reduced ascorbate and glutathione were measured in the two genotypes during the warming period following chilling. This is of interest given the rapid changes in ascorbate and glutathione levels and redox state shown in **Fig. 3** and also because the line IL9.2.5 has been previously shown to have increased MDHAR activity under certain conditions, which is correlated with fruit ascorbic acid levels (Stevens *et al*. 2008). The evolution of MDHAR activities following chilling are shown in **Fig. 4A**. Immediately following the end of the chilling period, similar MDHAR activities are found for both genotypes. MDHAR activity in M82 remains stable from 0 hours to 1.5 hrs, and decreases at 4 hrs. From 4 to 27 hrs, MDHAR activity remains unchanged. In the case of IL9.2.5, MDHAR activity increases just after the end of chilling then stabilizes from 1.5 hrs. The greatest difference in MDHAR activity between the two lines is seen after 4 hrs of warming. The evolution of DHAR activity, the second enzyme involved in regenerating reduced ascorbic acid, is shown in **Fig. 4B**. At 0 hrs, DHAR activity is higher in M82 than in IL9.2.5. This tendency is rapidly inverted and 1.5 hrs after the end of chilling DHAR activity in M82 decreased whereas in IL9.2.5 it increased. Between 1.5 and 27 hrs post-chilling, DHAR activity slowly declines in both genotypes.

The APX activity for the two genotypes is not significantly different up to 4 hrs (**Fig. 4C**). From 1.5 hrs post chilling, APX activity increased in IL9.2.5 whereas in M82 the activity started to decrease. From 4 to 27 hrs post chilling, it remained stable in IL9.2.5 and increased in M82, although the differences are only significant at 4 hrs. The GR activity for the two genotypes is not significantly different over the post-chilling period although IL9.2.5 seems to show a higher activity than M82 (**Fig. 4D**).

DISCUSSION

Modification of fruit metabolism and redox state following cold storage in M82 and IL9.2.5 fruits

The end of cold storage is followed by an increase in fruit temperature and re-exposure of the fruit to light, which is followed, as expected, by modifications of the fruit metabolism and especially of its antioxidant systems. The two genotypes show contrasting behaviour in terms of the response of these antioxidant systems: in IL9.2.5, the ascorbate-related enzyme activities increased in parallel with a rapid increase in the redox state of the ascorbate pool 0.5 hrs after the end of the chilling period. The glutathione pool exhibited a similar, but slightly delayed, evolution compared to the ascorbate pool, as the redox state in IL9.2.5 reached a maximum at 4 hrs post chilling. In contrast, M82 tomatoes showed a decrease in the ascorbate redox state 0.5 hrs post-chilling. As for IL9.2.5, the redox state of the glutathione pool of M82 tomatoes followed the ascorbate

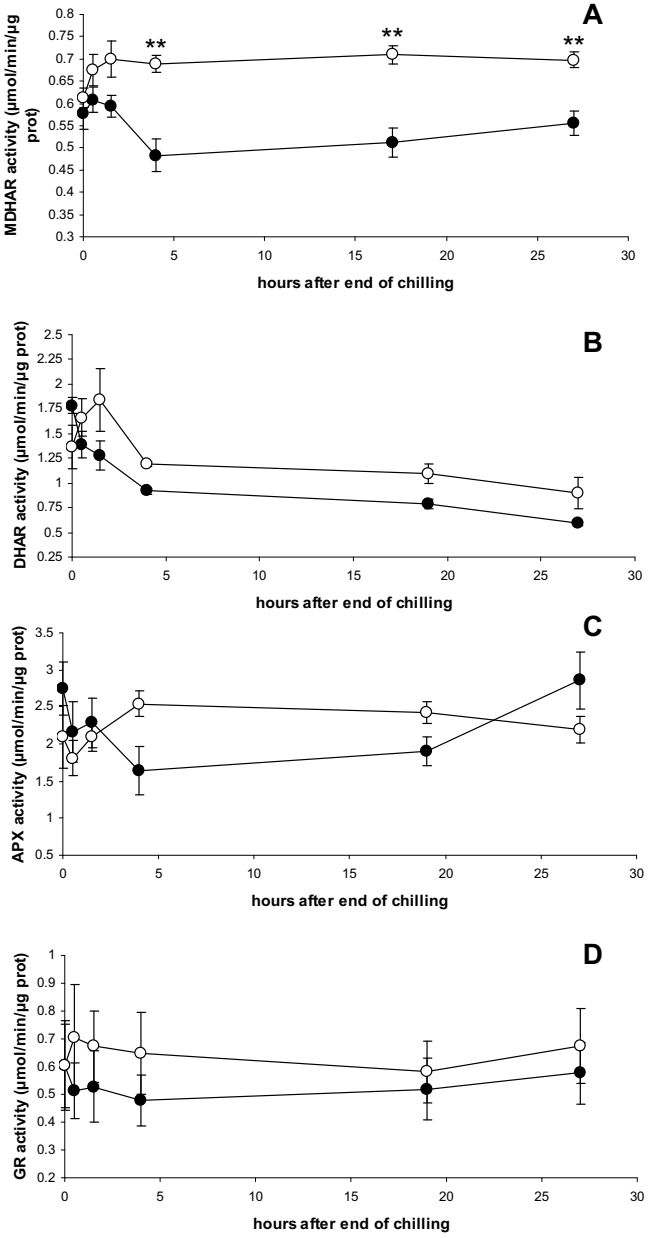

Fig. 4 Specific activity of the four enzymes of the ascorbate glutathione cycle in M82 fruit (solid circles) and IL9.2.5 fruit (open circles) at 0, 0.5, 1.5, 4, 19 and 27 hours after the end of chilling (26 days at 4°C). Results are expressed in µmol/min/µg protein. The values are averages from the two independent experiments. (**A**) MDHAR activity. (**B**) DHAR activity. (**C**) APX activity. (**D**) GR activity. Asterisks indicate significant differences (*: $p<0.05$; **: $p<0.01$).

redox evolution but with a slight delay, the redox state decreased to a minimum between 1.5 and 4 hrs post-chilling. The differences in redox state were associated with a significant loss of firmness between the two lines during the warming period, suggesting a link between the two. These observations are consistent with the hypothesis that chilling injury is linked to oxidative stress: Even though the link observed is correlative, it shows that a more efficient antioxidant system seems to provide a protective effect against oxidative stress and could be responsible for the diminution of chilling symptoms.

Pectin methyl esterase modifies esterified homogalacturonan components of pectin and is a critical enzyme for tissue remodelling and fruit maturation and therefore a link is often observed between fruit softening and PME activity (Jarvis *et al*. 2003; Brummell 2006; Derbyshire *et al*. 2007). In this study, we showed that both genotypes exhibit the same methanol content, suggesting a similar PME activity, and that this methanol content is stable over the warming period (**Fig. 2**). This observation would tend to demonstrate that PME is not responsible for the differences observed in fruit firmness and seems to exclude an enzymatic degradation of fruit cell walls during the warming period, as polygalacturonase (the enzyme responsible for pectin solubilisation and depolymerisation) is enhanced by an increase in PME activity (Pressey and Avants 1982; Wakabayashi *et al*. 2003). Fruit weight loss (which is due to fruit dehydration) has also been measured during the warming period (**Fig. 1B**) and no significant differences have been observed between the genotypes and during the warming period. These results show that fruit dehydration and PME activity are not responsible for the loss of fruit firmness observed during the warming period following chilling. Therefore an alternative hypothesis is that an increase in reactive oxygen species could be responsible for a non-enzymatic degradation of cell walls or cell membranes and could explain the increased loss of firmness observed in M82 compared to IL9.2.5, as depletion of the ascorbate and glutathione redox pools occurs in parallel with loss of firmness in M82 (i.e. at 1.5 and 4 hrs following the end of chilling).

Ascorbic acid content and recycling following cold storage

Introgression line IL9.2.5 has been obtained by introgressing a 8 cM fragment of *Solanum pennellii* into the genetic background of the M82 cultivar of *Solanum lycopersicum*. This fragment contains the wild species MDHAR allele and carries an ascorbic acid QTL (Stevens *et al*. 2007). We have demonstrated that loss of fruit firmness induced by storage at 20°C after chilling is higher in M82 than in IL9.2.5. These results indicate a better tolerance to the warming period following chilling for IL9.2.5 or a better tolerance of the chilling period which is manifested during the warming period. For example, the higher MDHAR activity and ascorbate levels observed in IL9.2.5 may have allowed an enhanced defence against oxidative stress in comparison to M82. These differences could explain the differences in the behaviour of the ascorbate-glutathione system between the two genotypes in response to warming and light exposure: while the system can be activated in IL9.2.5, it is already overcome by oxidative stress in M82 and is therefore less efficient. As the importance of oxidative stress has been highlighted many times in the chilling injury phenomena (Sala 1998; Toivonen and Sweeney 1998; Hodges *et al*. 2001; Wismer 2003), it seems clear that these differences may be responsible for the unequal appearance of firmness-related chilling injury symptoms in the two genotypes.

Changes in the pool of ascorbic acid can be a result of increased synthesis or a steady rate of synthesis and an increase in the regeneration of the oxidised form, considering that this form is likely to be degraded and lost under conditions where ascorbate is being oxidised. After chilling and during the warming period, the changes occurring in the pool of reduced ascorbic acid are more pronounced and more rapid than the changes in the total ascorbic acid pool when both genotypes are compared, as evidenced by the changes in the redox state of the pool. This indicates the importance of the regeneration of the reduced form of ascorbate from the oxidised form i.e. the redox state of the ascorbate pool. The 'increased regeneration' hypothesis is consistent with the increase in MDHAR and DHAR activities observed, which increased rapidly in IL9.2.5 in line with the reduced ascorbic acid content. In contrast, in M82, the end of cold storage is rapidly followed by a decrease in reduced ascorbic acid levels, while total ascorbic acid levels remain relatively unchanged, leading to a decrease in the redox state. The ascorbate pool is therefore highly oxidised in contrast to IL9.2.5 which is not surprising given the decrease observed for both MDHAR and DHAR activities in M82 after chilling.

In both M82 and IL9.2.5, MDHAR and DHAR activity followed a similar pattern post-chilling, indicating a pos-

sible co-regulation of these two genes. The rapidity of the response suggests that post-translational modification affecting enzyme activity may be occurring following exposure to light or increasing temperature. Post-translational modifications could include phosphorylation which is often associated with enzyme activation or deactivation. By studying the activity of these two enzymes under different conditions and in different genotypes, such correlations could be confirmed.

We have showed that the rapid changes in ascorbic acid levels in the two genotypes could be explained, at least in part, by differences in the ascorbic acid recycling pathway, but these results do not exclude a role for ascorbic acid synthesis.

Antioxidant systems and chilling injury

These results indicate an important role for the ascorbic acid recycling pathway and the glutathione pool in chilling injury phenomena, but do not exclude a role for other antioxidants. Apart from ascorbic acid, it has been shown that the main antioxidants in tomatoes are carotenoids and phenolic compounds (Giovanelli *et al*. 1999). It would be interesting to assay different antioxidant compounds under both chilling and normal conditions, in order to highlight changes occurring in the antioxidant profiles when tomatoes are exposed to a chilling stress. For instance, it has been shown that levels of lycopene (the carotenoid responsible for the red colour of tomatoes) were significantly greater in tomatoes stored at room temperature than in tomatoes stored at low temperature (Javanmardi and Kubota 2006). Measurement of lycopene levels in the two lines described in this study (M82 and IL9.2.5) could also be performed, as M82 tomatoes are less red after chilling than the IL9.2.5 ones (unpublished observation). Furthermore, as we showed that variation in ascorbic acid content has an influence on chilling tolerance, it would be interesting to determine if this is the case for other antioxidants.

In summary we have shown a rapid response of the antioxidant system in tomato to re-exposure to normal temperatures and light following chilling which parallels changes in fruit firmness. The ascorbate redox state responds rapidly and is therefore probably the first line of defence against oxidative stress in the fruit. The changes are followed by changes in the redox state of the glutathione pool. The response is contrasted between the two genotypes studied: fruits from IL9.2.5 (with the wild species alleles) have increased reduced ascorbic acid compared to the parent M82 where both antioxidant pools become highly oxidised. This observation is correlated with the activity of enzymes involved in ascorbate regeneration i.e. MDHAR and DHAR and reflects the ability of IL9.2.5 to withstand chilling as shown by measurements of fruit firmness.

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