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Decreased monodehydroascorbate reductase activity reduces tolerance to cold storage in tomato and affects fruit antioxidant levels

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ABSTRACT

Fruit antioxidants are necessary for human health and also have physiological roles in protecting fruit against oxidative stress. Our previous work has shown that monodehydroascorbate reductase (MDHAR) activity was positively correlated with fruit resistance to cold storage in introgression lines of tomato, *Solanum lycopersicum*. We have produced transgenic lines with reduced MDHAR activity to directly study the impact of this gene on fruit resistance to chilling and the antioxidant pool in two different genetic backgrounds: M82, a processing tomato with low to medium ascorbate levels, and IL925, an introgression line of M82 containing a fragment of the wild tomato *Solanum pennellii* genome, including the *S. pennelli* MDHAR allele. Large decreases in fruit MDHAR activity were obtained and correlated with slight losses in fruit firmness and fruit ascorbate, an effect that was independent of the genetic background. Fruit colour after chilling was also positively correlated with the redox state of the ascorbate pool and fruit firmness, although at harvest these correlations were less significant. Furthermore, other modifications in fruit of transgenic lines were observed as red fruit showed increased glutathione levels. Therefore manipulation of MDHAR activity confirms the link between antioxidant protection, fruit tolerance to chilling and fruit ripening at low temperatures, although the strength of the phenotypes indicates that other factors are involved.

1. Introduction

Fruit such as tomato are chilling sensitive and storage of fruit below temperatures of 10 °C provokes symptoms of chilling injury. The majority of these symptoms usually appear after cold storage, when fruit are returned to light and normal ripening temperatures; they include irregular colouration, loss of fruit firmness, fruit deliquescence and increased susceptibility to postharvest decay (Jackman et al., 1988; Saltveit and Morris, 1990). The appearance of these symptoms suggests that physiological processes, such as membrane and cell wall de-structuration, pigment bleaching or altered maturation occur in chilled fruit. As cold storage is an efficient way of extending fruit shelf-life and protecting against pathogen attack during the storage period, it is still widely used in the fruit industry. Therefore, genetically controlled parameters affecting chilling resistance have been extensively sought, as the selection of chilling resistant fruit allows long storage without loss of fruit quality.

It was first suggested that membrane damage was at the centre of the chilling sensitivity process, and correlations have been observed between the level of unsaturation of membrane lipids and chilling sensitivity (Lyons and Raison, 1970; Nishida and Murata, 1996). Chilling is also thought to deregulate the normal ethylene-induced ripening process, and it should be noted that the phenotypes used to judge chilling injury, such as fruit colour and firmness, can be ethylene-dependent. However, mutant studies have revealed that climacteric ethylene is not essential for initiating chilling injury in tomato, demonstrating that other factors are determinants in chilling injury (Cheng and Shewfelt, 1988; Lurie et al., 1996; Luengwilai and Beckles, 2010; Page et al., 2010). Progressively, a link has been established between reactive oxygen species, antioxidants and chilling injury. Cold stress has been shown to enhance the activity and transcript levels of many antioxidant enzymes as well as H_2O_2 accumulation in cells (Saruyama and Tanida, 1995; O'Kane et al., 1996; Sato et al., 2001; Kuk et al., 2003). More recently, studies on lines with different ascorbate levels revealed that efficient antioxidant protection before and after

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cold storage of tomato fruit was negatively correlated with chilling injury (Stevens et al., 2008; Gest et al., 2010). Correlations such as these have also been found in apple (Davey and Keulemans, 2004; Davey et al., 2007). These links between the presence of antioxidants and protection against chilling injury can be explained in part by the fact that (i) H_2O_2-dependent oxidative processes contribute to fruit softening (Brennan and Frenkel, 1977; Fry et al., 2001), (ii) cold stress is known to deregulate photosynthesis and respiration, and can therefore lead to generation of reactive oxygen species (ROS) by energy transfer to oxygen (Suzuki and Mittler, 2006), and (iii) both ROS and antioxidants are involved in fruit ripening, a process that is altered in chilling injury (Jimenez et al., 2002). Therefore the production of ROS within the fruit might be a primary event in chilling stress and associated symptoms, explaining why elevated antioxidant systems induce better chilling protection.

Fruit antioxidants include both lipid soluble (α-tocopherol and carotenoids) and water soluble components (ascorbate, glutathione, polyphenols). The redox status of cells is determined by both ascorbate and glutathione levels, although changes in the glutathione redox state may be more closely linked to central metabolism (Foyer and Noctor, 2011). Glutathione, together with the enzyme necessary for its efficient recycling, glutathione reductase (GR), are therefore crucial for the maintenance of redox homeostasis, especially under chilling conditions (Kocsy et al., 2001). However, glutathione levels are much lower in fruit such as tomato, compared to ascorbate levels, and this latter molecule therefore represents a major fruit antioxidant which may act as a first line of defense in protection against chilling stress, before the glutathione pool is involved (Gest et al., 2013b). In tomato, post-chilling redox changes in the ascorbate pool occur rapidly following a return to normal temperatures, and redox changes in the glutathione pool were shown to occur after the changes occurring in the ascorbate pool (Gest et al., 2010). Enzymes involved in restoring the redox state of the glutathione and ascorbate pools such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and GR could therefore play a key role in protection against chilling injury and maintenance of postharvest fruit quality. For example, DHAR may be involved in flesh browning in apple (Mellidou et al., 2012). Correlations between an oxidized ascorbate pool and flesh browning have been shown to exist in both apple and pear (Veltman et al., 1999; Davey et al., 2006).

An MDHAR gene in tomato was previously identified as being a candidate for an ascorbic acid QTL and as having a role in fruit chilling tolerance (Stevens et al., 2007, 2008). This gene has also been shown to encode a cytosolic and peroxisomal isoform of MDHAR involved in the light-dependent regulation of the ascorbate pool (Gest et al., 2013a). To test the role of this gene in chilling tolerance we have produced RNA interference (RNAi) lines in a processing tomato background (M82) and in an introgression line containing a quantitative trait loci (QTL) for fruit ascorbate due to the presence of tomato alleles from the wild *Solanum pennelli* species on chromosome 9 (IL925). The line IL925 has already been shown to exhibit higher ascorbic acid levels and higher MDHAR activity under certain conditions, and enhanced resistance to chilling injury (Stevens et al., 2007, 2008; Gest et al., 2010). The aims of this study were therefore to evaluate the impact of a modification of MDHAR activity in these tomato genotypes on both fruit firmness following chilling and the fruit antioxidant pool under postharvest conditions.

2. Materials and methods

2.1. Tomato genotypes

IL925 is a previously described introgression line derived from a cross between the wild tomato accession *Solanum pennellii* LA716 (*S. pennellii*) and M82, a processing tomato variety with determinate growth (Eshed and Zamir, 1995). Introgression of wild *S. pennellii* alleles, including MDHAR3, into an M82 background, increases fruit ascorbate content (Stevens et al., 2007).

2.2. Generation of RNAi monodehydroascorbate reductase lines in M82 and IL925

Hairpin RNA constructs were designed for silencing MDHAR by an RNA interference (RNAi) strategy as previously described (Gest et al., 2013a). The MDHAR gene corresponds to a previously identified gene on chromosome 9 (MDHAR3, SGN-U573751, bin9D (Stevens et al., 2007)) which encodes a cytosolic and peroxisomal isoform of MDHAR (Gest et al., 2013a). The RNAi construct used has been shown to specifically target this MDHAR isoform as the expression of the two other MDHAR isoforms in tomato does not change in RNAi lines (Gest et al., 2013a). M82 tomato cotyledons were transformed based on a previously described method (Hamza and Chupeau, 1993). Leaf tissue was tested for ploidy by flow cytometry (by use of a Ploidy Analyser, Partec, Germany, according to the manufacturer's instructions) and for the presence of the transgene by PCR. Plants not containing the transgene and non-diploid plants were eliminated. Eighteen independent lines were produced of which three (M82-16md, M82-20md and M82-39md) were selected based on the reduction in MDHAR activity in fruit. Segregation analysis showed that lines M82-20md and M82-39md had only one copy of the transgene whereas M82-16md had a least two copies. In order to introduce the same transgene into IL925, individual crosses were carried out between the three M82 transgenic lines and IL925 generating plants heterozygous for the IL925 locus. Selection on kanamycin allowed elimination of plants not containing the transgene. Self-pollination of these plants generated 25% homozygous IL925 plants. The homozygous IL925 plants were selected for using two PCR markers as described below.

2.3. Genotyping of the IL925 transgenic lines

Two cleavage amplified polymorphism markers were generated based on DNA sequence differences between *S. pennellii* and *Lycopersicon esculentum*. DNA was isolated from the leaves of individual plants and PCRs carried out using standard conditions and the following primers:

1. 415F 5′-GGGCAAGTTGAAGAGGAGAAGGG and 415R 5′-GCAGGGGGTTGAACCTTTGC amplified a fragment of 443 bp. A *Hin*fI site identified in the *S. pennellii* sequence, which was absent from the M82 sequence, generated a direct cleavage amplified polymorphism marker. Digestion of the PCR product produced two fragments if the *Hin*fI site in the *S. pennellii* sequence was present.
2. 345F 5′-AAACCAGAGACGTTGAAACAA and 345R 5′-TTAGCTATTTGCCGGATGATA amplified a fragment containing an additional *Bcc*I site in *L. esculentum.*

The use of these two markers in combination allowed detection of plants homozygous for the IL925 region. In one case (IL925-20md), only plants heterozygous for the IL925 region were recovered and so interpretation of results from this line should take this factor into account.

2.4. Plant growth conditions and sampling

Plants were grown in a multispan Venlo-type greenhouse, orientated N-S in 4 L pots (potting compost P3 Tref, Tref EGO substrates BV). Plant nutrition and chemical pest and disease control were in accordance with commercial practices. Water was supplied to the plants using a drip irrigation system to maintain 20–30%