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# Variation in Tomato Fruit Ascorbate Levels and Consequences of Manipulation of Ascorbate Metabolism on Drought Stress Tolerance

V. Truffault<sup>1,2</sup>, N. Gest<sup>1</sup>, C. Garchery<sup>1</sup>, M. Causse<sup>1</sup>, R. Duboscq<sup>1</sup>, G. Riqueau<sup>1</sup>, C. Sauvage<sup>1</sup>, H. Gautier<sup>2</sup>, P. Baldet<sup>3</sup> and R. Stevens<sup>a</sup>

<sup>1</sup> INRA, UR1052, Génétique et Amélioration des Fruits et Légumes, Domaine St Maurice, 84143 Montfavet, France

<sup>2</sup> INRA, UR1115, Plantes et Systèmes de Culture Horticoles, Domaine St Paul, Site Agroparc, 84914 Avignon, France

<sup>3</sup> INRA, UMR1332, Biologie du Fruit et Pathologie, 33883 Villenave d'Ornon, France

**Keywords:** water, diversity, adaptation, growth, *Solanum lycopersicum*, vitamin C

## Abstract

Tomato is an important crop worldwide and one of the major sources of vitamin C (ascorbate) in the human diet. Ascorbate contributes both to tomato nutritional quality and has roles in stress tolerance and adaptation to the environment. In this study we show the variability that exists in tomato germplasm in terms of ascorbate content (10 to 90 mg/100 g fwt) which could be starting point for evaluating correlations with physiological traits potentially linked to ascorbate. We have then manipulated genes involved in ascorbate metabolism, using RNA interference, to investigate their influence on fruit ascorbate levels, fruit physiology and yield under both normal and drought stress conditions. There is some evidence that one of the genes chosen affects fruit size and yield under different conditions, but clear effects on the fruit ascorbate pool are not seen. We conclude that ascorbate metabolism is complex and can also have wider effects on fruit physiology and growth.

## INTRODUCTION

Crops showing tolerance and maintenance of yield following prolonged periods of mild or severe stress, particularly drought stress, are necessary for world food production (Morison et al., 2008). Drought stress seriously limits plant and crop productivity worldwide and is one of the major abiotic stresses that represent the primary cause of crop loss worldwide, causing average yield losses of 50% for major crops (Boyer, 1982). In order to develop plants adapted to drier conditions, understanding of the physiological and molecular responses to water limitation is required. The impacts of water limitation on plant physiology are rapid and numerous and include changes in stomatal conductance and decreased growth and photosynthetic levels. The effects of water limitation on photosynthetic efficiency lead to decreases in carbon fixation and therefore ultimately a drop in plant, grain or fruit yield. At a molecular level, drought stress induces signals leading to alterations in gene expression, accumulation of abscisic acid, synthesis of drought-responsive proteins and metabolites, often over the course of several days or months. Ideally, traits should allow the prolonged maintenance of photosynthesis and include other factors that contribute to yield: e.g., increased mobilisation of carbon supplies from source to sink or improved root architecture (Prudent et al., 2009, 2010; Muller et al., 2011). Antioxidants, such as ascorbate, can play roles in the processes affecting drought tolerance (Garchery et al., 2013).

Ascorbate (vitamin C) was initially purified by Dr. Szent-Gyorgyi from pepper, a fruit rich in ascorbate, in the early 20<sup>th</sup> century. Dr. Szent-Gyorgyi was subsequently awarded a Nobel prize in 1937 due in part to his work on the purification of this molecule. The history of the discovery of ascorbate however dates back to 1747 when Sir James Lind, a Scottish doctor in the British navy, carried out an experiment on sailors at sea in what was a very early example of a controlled trial. Sailors living at sea for long

<sup>a</sup> Tel: + 33 4 32 72 27 18; Fax: + 33 4 32 72 27 02; e-mail: Rebecca.stevens@avignon.inra.fr

periods fell ill from an incurable illness called scurvy and Lind gave them one of six treatments, one of which was two oranges and a lemon. The fruit gave the best results and the subsequent addition of lemon juice to the diet of sailors rid the Royal Navy of scurvy by the end of the 18<sup>th</sup> century.

Plants and fruit are major sources of ascorbate, but concentrations of this molecule are highly variable from one species to another and in different tissues, fruit showing a particularly good range of variability. Citrus fruit (*Rutaceae* family) are reputed for being rich in ascorbate, they contain around 50 mg/100 g fw, but fruit such as the camu-camu (*Myrciaria dubia*; an Amazonian berry; 2-3 g/100 g fw) and acerola (*Malpighia emarginata*; 1 g/100 g fw) are so far the richest sources found. In regularly consumed fruit such as tomato (*Solanum lycopersicum*), concentrations are lower: 10-20 mg/g fw for large-fruited domesticated cultivars (Stevens et al., 2007; Gest et al., 2013b).

As well as being an essential vitamin for the human diet, ascorbate in plants has many cellular functions, mostly linked to the molecule's capacity to donate electrons. The molecule is a cofactor for numerous enzymatic reactions such as for the oxygenases involved in the synthesis of hormones, flavonoids and alkaloids (Prescott and John, 1996). In chloroplasts, ascorbate is necessary for the xanthophyll cycle which allows dissipation of light energy as heat (Demmig-Adams and Adams, 1996). The molecule also has a role in growth and development, including aspects such as flowering time, as study of the *Arabidopsis* low vitamin C (*vtc*) mutants has shown (Pavet et al., 2005; Dowdle et al., 2007). Finally numerous studies have linked ascorbate, or ascorbate recycling, to stress tolerance in different species (Huang et al., 2005; Yamamoto et al., 2005; Stevens et al., 2008; Garchery et al., 2013).

At physiological pH, ascorbic acid is present in its mono-anionic form, ascorbate, with the chemical formula  $C_6H_8O_6$ . The molecule is hydrosoluble and the presence of an enediol group gives the molecule its electron donating properties. In losing electrons, the ascorbate molecule becomes oxidized and is found either as a monodehydroascorbate radical (one electron lost) or as the more stable form of dehydroascorbate (DHA; two electrons lost) (Fig. 1). The ratio of the oxidized to the reduced forms of ascorbate fluctuates according to environmental conditions or during plant development. As ascorbate is an unstable molecule it is rapidly oxidized to dehydroascorbate, which is also unstable, and is degraded to produce intermediates such as oxalate and threonate (Green and Fry, 2005). Ascorbate levels are under both genetic and environmental control and are in particular controlled by light (Gautier et al., 2009). The major genetic control points that are known about in plants are the genes controlling the synthesis and recycling of ascorbate (Fig. 2). *GDP L-galactose phosphorylase* appears to exert most of the control of the flux through the synthesis pathway (Bulley et al., 2009) and the expression of this gene is often correlated with ascorbate content (Massot et al., 2012). Reductases such as monodehydroascorbate reductase and dehydroascorbate reductase regenerate ascorbate from mono- or dehydroascorbate and therefore have roles in stress tolerance (Kwon et al., 2003; Eltayeb et al., 2007; Stevens et al., 2008; Gest et al., 2010, 2013). In this paper we examine ascorbate variability in tomato fruit germplasm and show how the manipulation of two genes involved in ascorbate metabolism can affect fruit physiology under both normal conditions and abiotic stress conditions. The results may give help in directing future research on ascorbate and its functions and also for breeding in tomato.

## MATERIALS AND METHODS

### Plant Material

In tomato, a core collection maximizing the genetic diversity with a minimum of individuals has been built for different purposes including population genetics, investigation of domestication or association mapping (Ranc et al., 2008). This collection consisted of 360 accessions made up of domesticated cultivars (*S. lycopersicum*), cherry tomato cultivars (*S. l. cerasiforme*) and wild accessions (*S. pimpinelifolium*). A subset of this collection that contained 19, 32 and 148 wild, domesticated and cherry accessions

respectively was used in the present study. The *Solanum lycopersicum* L. cultivar ‘West Virginia 106’ (WVa106, a cherry tomato) accession was chosen for transformation for study of genes involved in ascorbate metabolism.

### **Standard Plant Growth Conditions and Sampling**

WVa106 plants were grown in a multispans Venlo-type greenhouse, orientated N-S in 51 pots (potting compost P3 Tref, Tref EGO substrates BV) in either spring (May harvest) or autumn (November harvest) in southern France. 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% drainage. Light intensities of 300-700 photosynthetically active radiation (PAR) were obtained over the culture period, a maximum of 700 PAR was obtained on sunny days. Flowers were mechanically pollinated three times a week and side shoots removed as they appeared. Plant material was harvested at solar noon on a sunny day unless otherwise stated. Harvested material was immediately frozen in liquid nitrogen and stored at -80°C. Prior to the molecular and biochemical analysis plant material was ground in liquid nitrogen. For all physiology experiments a minimum of 5 plants, making 5 biological replicates, per genotype and per condition were used.

### **Growth of Plants under Drought Stress Conditions**

To test the impact of drought stress on yield and fruit development (fruit size and ascorbate levels), plants were divided into two equally sized groups (at least 5 plants per genotype). One group was watered normally (maintaining one third drainage) whereas the second group was subjected to water stress by stopping watering for between 24 and 48 h, or until plants wilted completely, whichever was the sooner. The water stress was applied once a week for four consecutive weeks during fruit ripening on trusses 2 to 5. The stress was monitored by application of sensors to the base of the tomato plant stems linked to a recording device which monitored stem diameter throughout the period of the experiment. Fruits for analyses were harvested directly following the fourth and final application of drought stress.

### **Construction of RNAi Plasmids, Tomato Transformation and Selection of Lines**

RNAi plasmids for two genes involved in ascorbate metabolism, gene “D” and gene “O” were constructed. Fragments of 500-600 bp were amplified with specific primers (corresponding to the coding region of the two genes) containing Gateway adaptors from cDNA purified from tomato leaf RNA using standard techniques. The PCR products obtained were cloned into vector pDONR<sup>TM</sup>201 (BP reaction, Gateway according to the manufacturer’s instructions) and afterwards into the destination vector pK7WIWG2(1),0 (Karimi et al., 2002) (LR reaction, Gateway according to the manufacturer’s instructions). These constructs were used to transform *Agrobacterium* strain GV3101. WVa106 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 the flow cytometer ‘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. The lines produced concerned two genes involved in ascorbate metabolism (Fig. 2): lines D1 and D2 were independent lines for the same gene and line O1 represented another gene involved in ascorbate metabolism. In all cases, the reduction in expression of the specific gene was checked by RT-PCR or Q-PCR and both genes were found to be significantly under-expressed compared to wild type levels (data not shown).

### **Ascorbic Acid Content**

Measurements of ascorbic acid content were carried out as described (Stevens et al., 2006) on material conserved at -80°C. Extractions and assays were carried out in ice-cold 6% TCA. The microplate assay used was a spectrophotometric assay based on the

detection of dipyriddy- $\text{Fe}^{2+}$  complexes following the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by the reduced form of ascorbate present in the samples and comparison with standards of known concentrations. Total ascorbate (reduced + oxidised forms) was measured by mixing the sample with 5 mM DTT, to reduce dehydroascorbate, prior to the assay. Non-specific background fluorescence was eliminated by measuring the absorbance obtained by replacing the DTT with 0.3 U ascorbate oxidase. The efficiency of the oxidation was checked on ascorbate standards treated in the same way. All assays were carried out in triplicate.

## **RESULTS**

### **Variability in Tomato Fruit Ascorbate and Dehydroascorbate Content**

Fruit are a major source of vitamin C, particularly fruit that are regularly consumed such as tomato. However, within germplasm of different species, a wide range of variation in vitamin C levels can be found, which will have an impact on nutritional value and stress tolerance. Ascorbate and dehydroascorbate levels were therefore measured in red or ripe tomato fruit from the core collection described in the Materials and Methods. The results are shown in Figure 3. Tomato fruit ascorbate content and dehydroascorbate (DHA) content are variable in the collection both showing a normal distribution. Fruit ascorbate content shows a range of values of between 10 and 90 mg/100 g fwt for ascorbate (Fig. 3A) whereas DHA levels vary between 0 and 15 mg/100 g fwt (Fig. 3B): the low DHA values reflecting the fact that tomatoes were harvested when ripe when the ascorbate pool is almost fully reduced and DHA levels are low.

### **Application of Drought Stress to Wild Type and Transgenic Plants for Genes Involved in Ascorbate Metabolism**

As ascorbate is not only a nutritional marker but can play a protective role in plant physiology, particularly under conditions of stress, transgenic plants with altered ascorbate metabolism were produced and subjected to repeated drought stress as described in the Materials and Methods and compared with plants grown under normal conditions. The stem diameter measurements shown in Figure 4 show the fluctuations in stem diameter for a typical wild type plant and a typical plant from each of the transgenic lines (D1, D2 and O1) under control or drought stress conditions. The decrease in stem diameter occurs for all four types of plants during the four points of stress application while it does not appear in the control plants showing that the stress was effective.

### **Effect of Changing Expression of Genes Involved in Ascorbate Metabolism and Imposing Drought Stress, on Fruit Ascorbate Content**

Ascorbate content may fluctuate during drought stress or as a result of the transgenic manipulations. Ascorbate and dehydroascorbate levels were therefore measured in ripe fruit grown under control conditions or under drought stress. The results are shown in Figure 5. The alteration of the expression of different genes involved in ascorbate metabolism (transgenic lines D1, D2 and O1) did not have a significant effect on fruit ascorbate content either under control conditions or under the conditions chosen for the repeated drought stress (Fig. 5A). Similar results were obtained for fruit DHA levels (Fig. 5B).

### **Under-Expression of Genes Involved in Ascorbate Metabolism: Effects on Fruit Size and Yield under Control Conditions and Following Drought Stress**

Drought stress can have a negative impact on assimilate accumulation and therefore reduce fruit number, size or yield. Fruit size and yield were measured in the wild type and transgenic lines under the two conditions and results are shown in Figure 6. The under-expression of one of the genes involved in ascorbate metabolism has an effect on fruit weight, decreased average fruit weight is seen under both normal and stress

conditions for the transgenic lines D1 and D2 (Fig. 6A) whereas the line O1, under-expressing a different gene involved in ascorbate metabolism, does not show differences in fruit weight compared to wild type (WT) or the transformed control (WTt). Fruit weight decreases in all lines following drought stress but the effects are the largest for the small fruited lines D1 and D2 which show average decreases in fruit size of 15 and 22% respectively, compared to 9% for the wild type and 4% for the transgenic line O1. The effects of drought stress on total fruit yield were not significant but the transgenic lines D1 and D2, which have smaller fruits, also show smaller yields compared to the control lines (WT and WTt) and the line O1, under-expressing a different gene involved in ascorbate metabolism.

## DISCUSSION

Understanding the mechanisms governing plants' adaptation to the environment is a crucial challenge in the light of current issues concerning climate change. The ecological diversity present in populations and germplasm is a vital source of traits and alleles, many of which may have been inadvertently lost during domestication, which enable the plant to adapt to a changing environment, particularly in crop species. As wild species often show more tolerance to environmental stress than their cultivated relatives (Gur and Zamir, 2004), the exploitation of genetic diversity available in tomato is important to develop an understanding of the mechanisms that influence adaptation. In addition it can be used to identify key alleles for future breeding programs, so that this diversity is harnessed to develop stress-tolerant crops in which yield may be maintained under unfavourable environmental conditions.

In this study we have shown that a wide range of ascorbate concentrations are present in a collection of tomatoes representing the global genetic diversity of this fruit (including domesticated cultivars (*S. lycopersicum*), cherry tomato cultivars (*S. l. cerasiforme*) and wild accessions (*S. pimpinelifolium*) that could be useful for breeding for stress tolerance, or improved nutritional quality and as an important source of information necessary for understanding the links between ascorbate content and physiological functions in fruit.

Antioxidants such as ascorbate are an essential part of the plant's stress tolerance mechanisms and given the multiple roles of this molecule in plants, can act at different levels. Genes involved in ascorbate metabolism have already been shown to have roles in controlling stomatal conductance (Chen and Gallie, 2004; Fotopoulos et al., 2008; Garchery et al., 2013) for example. In this study, one of the two genes chosen has effects on fruit weight particularly under drought stress conditions. More work is required to understand at what level these genes are acting (for example in terms of photosynthesis, carbon fixation and carbon transport to fruit). The effects in red fruits on ascorbate levels are not significantly different to wild type and so several hypotheses need to be tested: (i) the impact of the under-expression of the chosen genes on ascorbate levels may be seen in other tissues; (ii) the plant has compensated for the reduced expression of these genes by activating other genes controlling ascorbate levels.

In a previously published study, links have been shown between the activity of an ascorbate oxidase enzyme and yield under different conditions, including water deficit (Garchery et al., 2013). The silencing of this isoform of ascorbate oxidase led to numerous phenotypic differences with wild type plants, including changes in stomatal conductance, sugar metabolism (hexose concentrations in leaves and fruit) and apoplastic ascorbate, sucrose and hexose levels. The expression level of different genes and the activity of enzymes involved in sugar metabolism and the source-sink relationship were also affected. Therefore the activity of enzymes controlling the ascorbate redox state could have wide roles including in carbon metabolism and processes controlling fruit yield under different conditions.

In summary, manipulation of ascorbate metabolism in tomato can have effects beyond being a marker for nutritional quality or stress tolerance traits, as manipulation of enzymes involved in ascorbate metabolism can also affect fruit growth and yield. The

diversity in terms of ascorbate metabolism, and probably activity of the enzymes involved in ascorbate metabolism, is therefore an extremely important resource and is useful for several reasons (i) as sources of new alleles to improve fruit nutritional value; (ii) as sources of useful alleles for stress tolerance and adaptation in tomato; and finally (iii) to help confirm or refute the correlations found in this study concerning drought tolerance, yield and ascorbate metabolism.

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## Figures

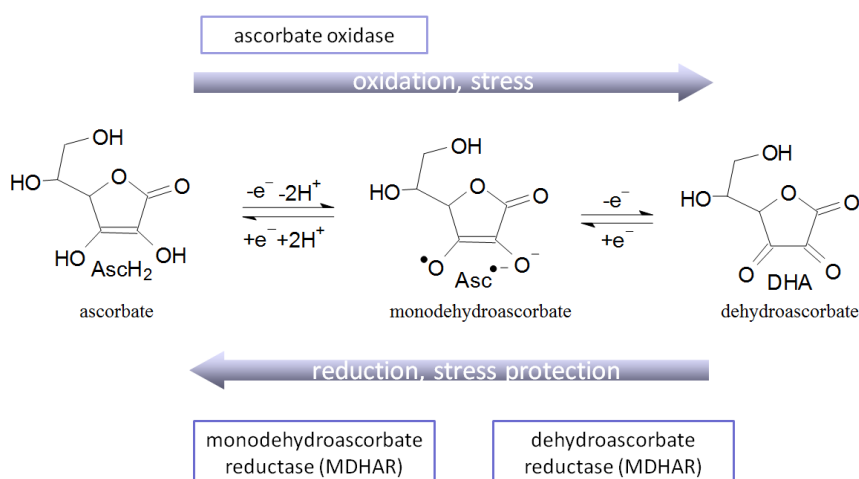


Fig. 1. The ascorbate molecule is unstable and will degrade into monodehydroascorbate (a radical, the first oxidised form) and dehydroascorbate (the second oxidised form). This degradation (oxidation) is linked with stressful environmental conditions or the activity of the ascorbate oxidase enzyme. Two ascorbate reductases (monodehydroascorbate reductase and dehydroascorbate reductase) catalyse the reduction of the oxidised forms back to ascorbate and thus replenish the ascorbate pool.

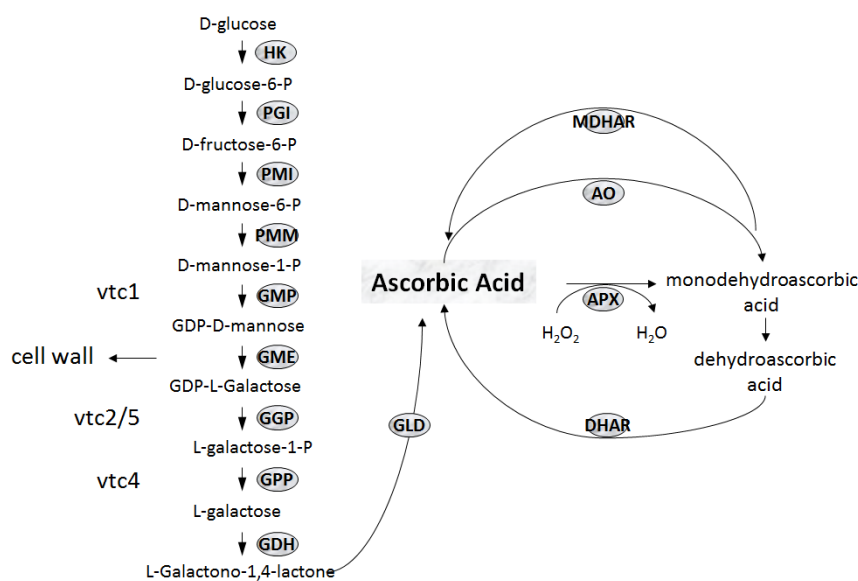


Fig. 2. The major pathways of ascorbic acid synthesis (left) and recycling (right) in plants. HK: hexokinase; PGI: phosphoglucose isomerase; PMI: phosphomannose isomerase; PMM: phosphomannose mutase; GMP: GDP-mannose pyrophosphorylase (vtc1); GME: GDP-mannose-3,5-epimerase; GGT: GDP-L-galactose phosphorylase or L-galactose guanyltrtransferase (vtc2/5); GPP: galactose-1-P phosphatase (vtc4); GDH: galactose dehydrogenase; GLD: galactono-1,4-lactone dehydrogenase; MDHAR: monodehydroascorbate reductase; AO: ascorbate oxidase; APX: ascorbate peroxidase; DHAR: dehydroascorbate reductase.

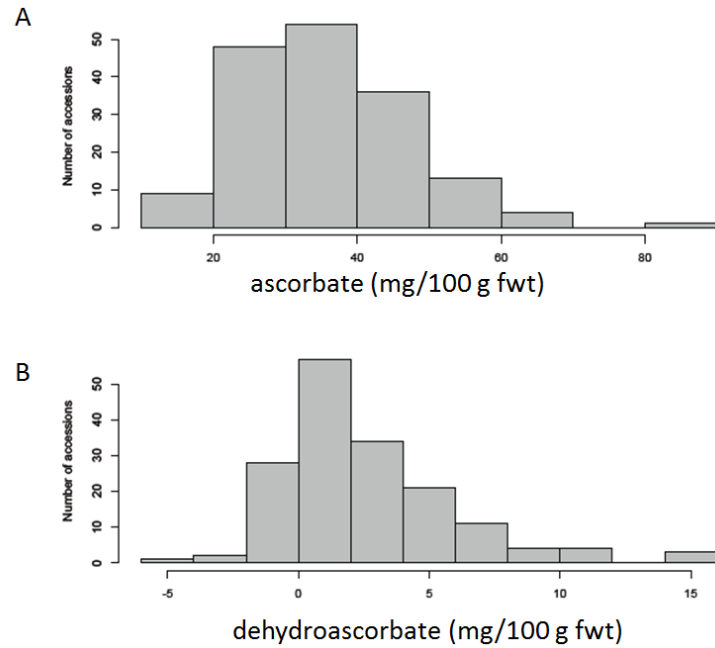


Fig. 3. The distribution of ascorbate and dehydroascorbate content in tomato germplasm (170 accessions made up of domesticated and cherry cultivars and wild accessions). Ripe fruit from the accessions grown in southern France in summer 2007 were harvested, ground and assayed for ascorbate and dehydroascorbate. The distribution of concentrations in mg/100 g fwt is shown. The negative values for DHA reflect the error present in the assay as little DHA is present in ripe tomato fruits, the ascorbate pool is mostly present in a reduced form.

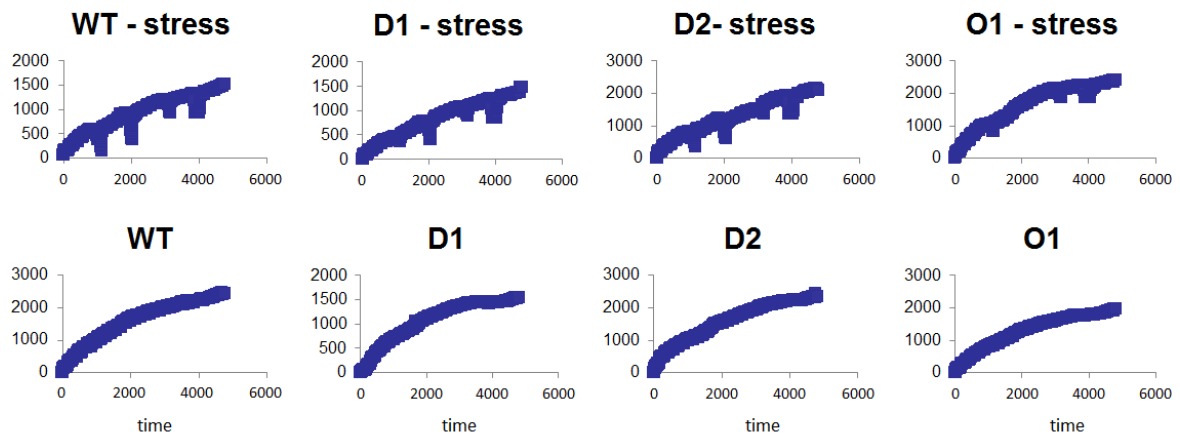


Fig. 4. Stem diameter measurements of plants under repetitive drought stress at weekly intervals (top row) or control plants (bottom row). Diameters of representative plants are shown (10 μm units). Measurements were taken and recorded every 10 min over the growing season (while trusses 2 to 5 were ripening). Data are shown for a wild type plant (WT) and the three lines under-expressing genes involved in ascorbate metabolism (lines D1, D2, O1).

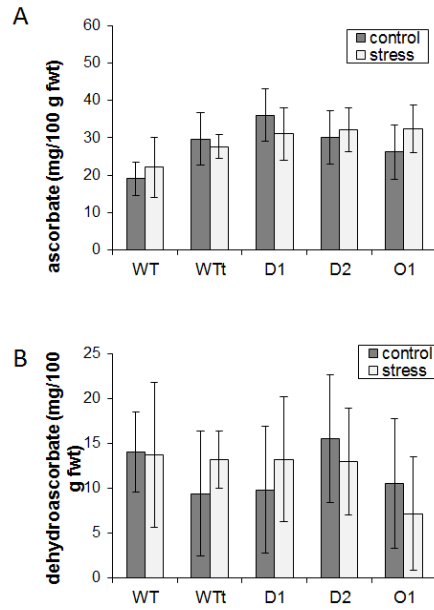


Fig. 5. Ascorbate and dehydroascorbate levels in red ripe fruit harvested at the end of the series of drought stress episodes from WT, transformed WT (WTt) and the three transgenic lines (D1, D2, O1). Control fruits were harvested at the same moment from plants that had not been subjected to drought stress. At least 5 plants per genotype per condition were used and a minimum of six fruits per plant were harvested.

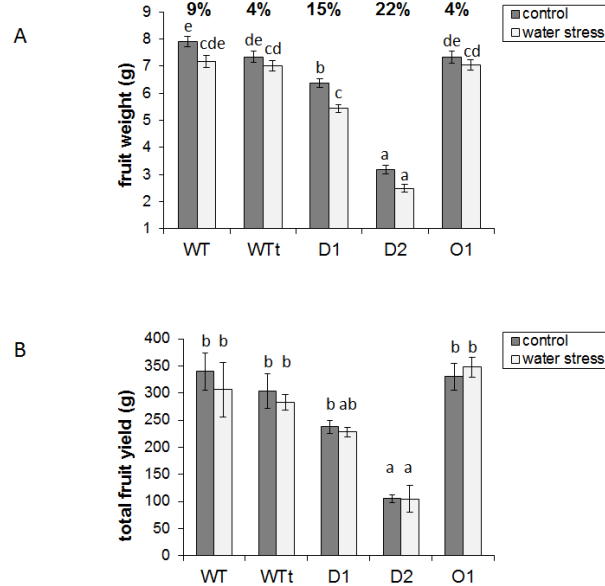


Fig. 6. Average fruit weight (all fruit) and total fruit yield of red ripe fruit harvested at the end of the series of drought stress episodes from WT, transformed WT (WTt) and the three transgenic lines (D1, D2, O1). Control fruits were harvested at the same moment from plants that had not been subjected to drought stress. At least 5 plants per genotype per condition were used. Statistical differences between samples were assessed using a Tukey test at 5%.