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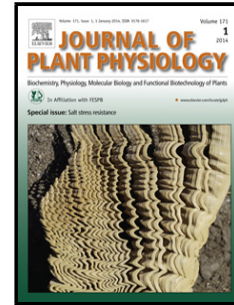
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## Is monodehydroascorbate reductase activity in leaf tissue critical for the maintenance of yield in tomato?

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### Summary

Ascorbate redox metabolism and growth have been shown to be linked and related to the activity of enzymes that produce or remove the radical monodehydroascorbate, the semi-oxidized form of ascorbate (ascorbate oxidase or peroxidase and monodehydroascorbate reductase respectively). Previous work in cherry tomato has revealed correlations between monodehydroascorbate reductase and ascorbate oxidase activity and fruit yield: decreased whole plant MDHAR activity decreases yield while decreased whole plant ascorbate oxidase activity increases yield under unfavourable environmental conditions. We aimed to investigate if similar effects on yield are obtained in a large-fruited variety of tomato, Moneymaker. Furthermore we wished to establish whether previously observed effects on yield in cherry tomato following changes in whole plant enzyme activity could be reproduced by reducing MDHAR activity in fruit only by using a fruit-specific promoter in cherry tomato (West Virginia 106). In Moneymaker, RNAi lines for monodehydroascorbate reductase did not show significant yield decrease compared to control lines when plants were grown under

optimal or non-optimal conditions of carbon stress generated by mature leaf removal. In addition, we show that a decrease in monodehydroascorbate reductase activity in fruit of cherry tomato had no effect on yield compared to a reduction in whole-plant monodehydroascorbate reductase activity: we therefore show that whole plant MDHAR activity is necessary to maintain yield in *cherry* tomato, suggesting that the carbon source in autotrophic tissue is more important than fruit sink activity. The present data also revealed differences between cherry and large fruited tomato that could be linked to a source of genetic variability in the response to monodehydroascorbate metabolism in tomato: maybe the domestication of tomato towards large-fruited lines could have affected the importance of MDHAR in yield maintenance.

**Abbreviations:** MDHA(R): monodehydroascorbate (reductase) DHA(R): dehydroascorbate (reductase) WT: wild type RNAi: RNA interference

**Keywords:** carbon limitation, ascorbate redox, *Solanum lycopersicum*, genetic background, yield

## Introduction

The redox status of plant cells influences plant growth and development through the network involving reactive oxygen species, antioxidants and hormones (Bartoli et al., 2013; Considine and Foyer, 2013; Kocsy et al., 2013; Schippers et al., 2016). As ascorbate is present in high concentrations in all cells, its redox status, which is maintained by the enzymes of the ascorbate-glutathione cycle, is important in controlling the redox state of the cell. Under optimal growth conditions, ascorbate and glutathione redox couples are maintained in a highly reduced state. The reduced form of ascorbate is oxidized, enzymatically or directly, into monodehydroascorbate (a radical; MDHA), with the loss of a single electron; dehydroascorbate (DHA) is then generated from the disproportionation of the radical form or the loss of a second electron from MDHA. Recycling of ascorbate is carried out by monodehydroascorbate reductase (MDHAR) which is an NADH-dependent enzyme and dehydroascorbate reductase (DHAR) which uses glutathione as an electron donor. Two DHAR genes have been mapped in tomato (Stevens et al., 2007) and MDHAR also belongs to multigene family as shown by the presence of three isoforms in tomato (Stevens et al., 2007) and six in *Arabidopsis* (Lisenbee et al., 2005). The isoforms, localized in different organelles,

can have different biological effects on global metabolism; the isoform targeted in this study, MDHAR3, is a cytosolic and peroxisomal enzyme (whereas MDHAR1 and MDHAR2 have been identified respectively in chloroplasts and in peroxisomes; (Gest et al., 2013; Haroldsen et al., 2011)). Dual targeting to both peroxisomes and cytosol has also been reported for glutathione reductase which is another ascorbate-glutathione cycle enzyme (Kataya and Reumann, 2010). It appears that chloroplastic MDHAR activity correlates positively with ascorbate content (Li et al., 2010), while MDHAR3 has shown negative correlations with ascorbate content (Gest et al., 2013; Haroldsen et al., 2011) in different genotypes.

Ascorbate and the activity of ascorbate recycling enzymes have been shown to be correlated to plant growth and yield. For example, in rice, over-expression of DHAR led to an improvement in terms of yield and biomass which was associated with an increase in photosynthetic activity, but also to better tolerance of environmental stress in the field (Kim et al., 2013). The authors suggested this was related to the co-activation of MDHAR, ascorbate peroxidase and glutathione reductase leading to higher ascorbate content and a less oxidized state of the ascorbate pool. High ascorbate content resulting from over-expression of the biosynthetic pathway genes also led to positive effects on growth and biomass in *Arabidopsis* (Lisko et al., 2013). It has also been shown that silencing of ascorbate oxidase activity improved yield in cherry tomato under unfavorable conditions (Garchery et al., 2013) and the opposite effect was seen on reducing MDHAR3 activity, again in cherry tomato: yield and vegetative growth were decreased with more pronounced effects being seen under conditions of carbon limitation (Truffault et al., 2016). At a cellular level, ascorbate metabolism and growth (cell division and expansion) are often closely related: the reduced form of ascorbate seems to stimulate mitosis (de Pinto et al., 1999; Liso et al., 1984) unlike DHA which is an inhibitor of cell cycle progression (Potters et al., 2000). It has also been shown that the radical MDHA stimulates cell expansion (Gonzalez-Reyes et al., 1994). Ascorbate oxidase activity also seems to stimulate cell expansion (Kato and Esaka, 2000) and the enzyme is suggested to regulate auxin levels at least in root meristems (Kerk et al., 2000).

These studies point to pleiotropic effects of the ascorbate molecule and its oxidized forms on both whole plant and cellular physiology. We have been particularly interested in how the ascorbate redox state controls growth processes, in particular yield, under different conditions. The genetic background is also a deciding factor in the establishment of yield as different genotypes have very different harvest indices and growth habits (determinate or

indeterminate). Domestication has increased the harvest index of many crops, including tomato (Tanksley, 2004), and in tomato has led to breeding of larger-fruited genotypes (Cong et al., 2002; Frary et al., 2000) typically with harvest indices of up to 60-65% (Ho, 1984; van der Ploeg et al., 2007). Cherry tomatoes represent an intermediate type of tomato between cultivated tomato (*Solanum lycopersicum*) and the closest wild ancestor (*Solanum pimpinellifolium*) and are probably the first domesticated form (Nesbitt and Tanksley, 2002; Ranc et al., 2008), their harvest index tends to be much lower compared to modern large-fruited varieties, presumably because of increasing sink strength of fruit during domestication.

We have previously identified differences in yield in cherry tomato when enzymes controlling ascorbate recycling are manipulated (Garchery et al., 2013; Truffault et al., 2016). The yield phenotypes appeared to be correlated to photosynthesis and sugar metabolism and particularly the environmental conditions. Our hypothesis was that carbon metabolism and/or translocation of sugars from leaves to fruits was affected in these plants. To test this hypothesis we developed two parallel experiments firstly we observed effects on yield in a large-fruited tomato where the harvest index (the ratio of total fruit yield to total plant biomass) is higher than in cherry tomato and therefore may be subject to different controls determining yield. For this the cultivated genotype Moneymaker was chosen. We studied the impact of modified ascorbate recycling in Moneymaker, under optimal growth conditions and conditions of carbon limitation, applied by leaf removal. Secondly we tested the hypothesis that sugar allocation was affected between leaves and fruit as previous results had been shown using plants with reduced whole-plant MDHAR activity. Thus, we investigated the modification of ascorbate recycling in fruits only as compared to the whole plant by using a fruit-specific promoter to control MDHAR silencing. We show that MDHAR activity does not significantly affect yield in Moneymaker. Also a reduction in fruit MDHAR activity does not affect yield in contrast to a reduction in whole plant MDHAR activity.

## Materials and methods

### Plant material

#### 1. Moneymaker

*Solanum lycopersicum* L. cv Moneymaker cotyledons were transformed using the MDHAR RNAi construct as previously described (Gest et al., 2013) (Solyc09g009390) and the AO

RNAi construct described in Garchery *et al.* (2013) (Solyc04g054690). The presence of the relevant transgene was verified by PCR. The six independent RNAi lines silenced for the MDHAR isoform used in this paper were MD-2MK, MD-3MK, MD-6MK, MD-25MK, MD-26MK and MD-29MK. Moneymaker RNAi lines for AO were labeled AO-2MK, AO-3MK, AO-4MK, AO-21MK, AO-22MK, AO-24MK. Lines studied were from the T1 generation, selected on kanamycin and were heterozygous or homozygous for the transgene and selected based on the reduction in enzymatic activity. Wild type plants were used as controls, WT\_MK1 and WT\_MK2, were from two separate seed bulks and treated independently during the experiments.

## 2. West Virginia 106

*Solanum lycopersicum* L. cv West Virginia 106 (cherry tomato) was transformed with the RNAi MDHAR construct (see above) described in Gest *et al.*, (2013). In the present study, the independent line under-expressing MDHAR3 is mds5. This line, conjointly with 2 others independent lines under-expressing MDHAR3 (mds3 and mds42), have been previously characterized in terms of MDHAR activity and yield (Truffault *et al.*, 2016). The RNAi fragment was also cloned into a Gateway<sup>TM</sup> compatible vector, pK8GWIWG-PPC2-B4, where the 35S promoter was replaced with a fruit specific promoter from the phosphoenolpyruvate carboxylase gene which is highly expressed during the phase of rapid fruit growth: this promoter has therefore been used to express or silence genes in tomato fruit as described previously (Fernandez *et al.*, 2009; Guillet *et al.*, 2012). Following transformation, measurements of MDHAR activity were carried out on fully-expanded leaves and red-ripe fruit of individual T0 plants. The lines were chosen based on the fact that they respected the following criteria (i) no change in leaf MDHAR activity compared to untransformed control lines (ii) a decrease in fruit MDHAR activity compared to untransformed or other control plants. On this basis we were able to select the following lines, the percentage fruit MDHAR activity compared to control (100%) is shown in brackets: Pmds1 (24%), Pmds4 (9%), Pmds5 (16%), Pmds6 (17%), Pmds14 (13%), Pmds15 (17%). T- is a non-transgenic segregating sibling fruit promoter plant. The non-transformed control line was labeled WT.

## Growth conditions and experimental design

### 1. Moneymaker

Moneymaker plants for the yield experiments were grown in a Venlo-type greenhouse located in Avignon (44°N), France. The experiment took place in Autumn 2012 in accordance with

commercial practice in terms of plant nutrition and pest control. Plants were potted into 5L-pots containing potting compost. Water was supplied to plants using a drip irrigation system to maintain 20-30% drainage. The day/night temperature in the greenhouse was maintained at 22°C/16°C and relative humidity at 70-80%. Flowers were mechanically pollinated three times a week and side shoots removed as they appeared. Total fruit yield was estimated by a precise counting of the number of fruit per truss and weight of the fruits from the first six trusses. For the leaf removal experiment (carbon limitation) only 1 leaf was kept per truss, other leaves were removed (see Figure 1) once fruit were set. 6 plants per condition and genotype were used as replicates.

## 2. West Virginia 106

West Virginia 106 seeds (pmds lines and the mds5 control RNAi line) were selected on 0.5 x Murashige and Skoog medium with 100 mgL<sup>-1</sup> kanamycin for the transgenic lines, and planted in potting compost in the greenhouse after 2-3 weeks under the same conditions as for the experiment with Moneymaker above. Total fruit yield was calculated per plant using a minimum of six plants per genotype. Fruits were harvested and counted once ripe using a maximum of three harvests. The average fruit weight was calculated per harvest and then per plant once harvesting was complete. The greenhouse culture of pmds lines was carried out between March and July 2017.

Fruits and leaves for biochemical assays and enzyme activity assays were quickly removed from the plants, fruit were cut and pericarps were selected and immediately frozen in liquid nitrogen and stored at -80°C before grinding to powder in liquid nitrogen. Harvesting of fruits and leaves was carried out at noon to minimise the effect of the hour of the day on ascorbate metabolism.

### Enzyme activity assays

MDHAR activity was measured in leaves from 3-week old plantlets or ripe fruits. The assay of the activity of MDHAR enzyme was based on the oxidation of NADH at 30°C. Extractions were performed on ground powder conserved at -80 °C in 600 µl of 50 mM Tris-HCl at pH 7.8. 50 µl of extract was mixed with 1 mL of 1 mM ascorbate, 0.2 mM NADH and enough ascorbate oxidase to give a linear production of MDHA radical. Measurements were performed at 340 nm in triplicate. Measures of MDHAR activity represent the sum of activities of different MDHAR isoforms. A least four biological replicates per point were used, except for the pmds T0 lines when only one plant was available.



AO activity was measured on leaves from 3-week old plantlets. The assay was based on a previously described method (Pignocchi et al., 2003). Leaf material (50 mg) was homogenized with 0.1 M sodium phosphate at pH 5.6, 0.5 mM EDTA. Then 100  $\mu$ L of extract was assayed rapidly at 265 nm in a final volume of 1 ml containing 0.1 mM ascorbate in the same phosphate buffer. The absorbance decrease at 265 nm was followed on addition of the extract. At least four biological replicates per point were used.

### **Ascorbate assay**

Measurements of ascorbic acid content were carried out as described (Stevens et al., 2006). Extractions were performed on ground powder conserved at -80 °C in ice-cold 6 % TCA. The spectrophotometric assay was based on the detection of dipyriddy-Fe<sup>2+</sup> complexes following the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> by the reduced form of ascorbate present in the sample. Total ascorbate content (reduced + oxidized forms) was measured by mixing the sample with 5 mM dithiothreitol to reduce DHA, prior to the assay. Each extract was measured in duplicate. The specificity of the assay has been checked by comparison with other known methods (Stevens et al., 2006) and by using ascorbate oxidase to remove all ascorbate in order to detect and deduce background interference. At least four biological replicates per point were used.

### **Sugar assay**

Measurements of glucose, fructose and sucrose were performed by HPLC, as previously described (Gomez et al., 2002). Sugars were extracted in a methanol:chloroform:water mixture (1/0.6/1) at 4 °C. Polyvinylpyrrolidone was used to trap phenols and extracts were then purified before quantitative analysis. Alternatively glucose, fructose and sucrose were measured in samples using a microplate method and the R-Biopharm kit (Saint-Didier-au-Mont-d'Or, France) according to the manufacturer's instructions.

### **Statistical analysis**

Data were analysed using 'R' software (R Development-Core-Team, version 3.2.5). The data presented in Figures and Tables were submitted to a one or two-way analysis of variance (ANOVA) followed by a Tukey or Fisher test (as defined in the legends) to group data from the transgenic lines and wild type based on significant differences. Groups sharing the same letter are not considered to be statistically different.

## Results

### MDHAR silencing in Moneymaker does not affect yield

Firstly to characterize the transgenic lines, MDHAR activity was assayed on young leaves of plantlets of Moneymaker MDHAR silenced lines and compared to wild type (WT\_MK). MDHAR activity was reduced in all RNAi lines except one (MD-3MK), by at least 50% for MD-2MK, MD-6MK, MD-25MK and MD-26MK (Table 1). Ascorbate oxidase activity of the independent Moneymaker silenced lines (AO-2MK to AO-24MK) was also assayed in plantlets. All lines except two (AO-2MK and AO-3MK) showed a significant reduction in AO activity (Table 2). We measured ascorbate and dehydroascorbate content in plantlets of Moneymaker AO and MDHAR RNAi lines (supplementary Tables S1 and S2). We observed an increase in ascorbate content in 4 out of 6 AO lines (AO-3MK, AO-4MK, AO-22MK and AO-24MK), and no significant changes in dehydroascorbate content except in AO-24MK. In the MDHAR RNAi Moneymaker lines, no lines showed a significant increase in ascorbate or dehydroascorbate compared to wild type (samples harvested at 13h on a sunny day).

The effect on yield, fruit size and fruit number of a reduction in MDHAR or AO activity in Moneymaker was studied under optimal growth conditions and conditions of carbon limitation (removal of two leaves out of three; Figure 1). Our previous publication using West Virginia 106 silenced for MDHAR showed that average fruit weight, and to a lesser extent yield, were reduced under control conditions and conditions of leaf removal compared to wild type (Table 3). In the case of Moneymaker MDHAR silenced lines, total fresh-weight fruit yield was not statistically different to wild type yield, when both wild type plants were considered, under optimal growth conditions (Figure 2A), and conditions of carbon limitation (Figure 2B). For each yield experiment, changes in average fruit weight are shown in Table 4 and the number of fruits per plant in Table 5. It is notable that the transformed plants showed a different response to wild type to carbon limitation as illustrated by comparing the lines under control and stress conditions (supplementary table S3). In this case we see that the yield ratio between the two conditions was higher than that the two wild types for all transgenic lines under-expressing MDHAR compared to the control condition. This observation is borne out by the statistical analysis which shows that four out of six of the MDHAR silenced lines were found in statistically different groups compared to control conditions.

Total fruit yield of Moneymaker AO silenced lines was surveyed under normal growth conditions and under the same conditions of carbon limitation described above. Under normal

growth conditions, Moneymaker AO silenced line AO-24MK was the only line showing differences in term of yield with the wild type (Figure 3 and supplementary Table S4), due to a reduced fruit weight (Table 6), the five other independent lines all showing yield that was not statistically different to either wild type group. When the plants were grown under a situation mimicking carbon stress, the only transgenic line to show a reduced yield phenotype was AO-21MK due to a combination of a lower fruit weight and a reduced number of fruits per truss (Table 7), none of the other five lines were different to wild type. When compared to previous work carried out in cherry tomato, these results highlight the genetic variability in the response of yield and ascorbate metabolism to MDHAR or AO activities. Thus, we went more deeply into possible explanations linked to the source-sink relationship using a fruit specific promoter.

### **In cherry tomato, modification of MDHAR activity induces changes in yield which are not correlated with MDHAR activity in fruit**

We studied the hypothesis that whole plant MDHAR activity was more important than fruit MDHAR activity in the establishment of yield by silencing MDHAR activity in fruit compared to the whole plant. In Figure 4, we see that total fruit yield per plant is unaffected in lines where MDHAR activity is silenced in fruit only (up to 9% of remaining fruit enzymatic activity, see Materials and methods), as no significant difference was apparent between these lines and controls (WT and T-). In contrast, MDHAR silenced line *mds5* showed a strong reduction in total fruit yield per plant (50% of WT): this is due to a smaller fruit weight (significant for *mds5*) and a lower number of total fruits (supplementary Table S5).

## **Discussion**

### **MDHAR activity does not affect yield in large-fruited tomato**

The process of domestication of tomato has been characterised by major changes in plant anatomy, notably a large increase in fruit size and weight which is apparently attributable to a few loci (Nesbitt and Tanksley, 2002). These loci often correspond to changes affecting the regulation of genes controlling flower anatomy: for example, carpel number or cell number (Chakrabarti et al., 2013; Frary et al., 2000) but plant hormones also affect fruit size (Ariizumi et al., 2013). Our previous work on cherry tomato has shown that slower early growth and a

decrease in yield are associated with MDHAR silencing and that such phenotypes were more pronounced under carbon limitation (Truffault et al., 2016). Differences in yield were also obtained with ascorbate oxidase silenced lines, which, in contrast to MDHAR, show improved yield under conditions of carbon limitation (Garchery et al., 2013). The average fruit weight of this genotype is less than 10g. The aim of the current study was to compare phenotypes in a large-fruited cultivated tomato variety for which we chose Moneymaker (average fruit weight above 50g). We suggest that the genetic background might play a role in the phenotypes as the harvest index (ratio of fruit yield to total crop weight) increases considerably when comparing a variety like Moneymaker with cherry tomato and therefore the mechanisms to distribute carbon between source and sink tissues are probably different.

Fruit yield of the Moneymaker transgenic lines was not different to wild type under normal growth conditions or carbon stress for either Moneymaker MDHAR or Moneymaker AO silenced lines (Figures 2 and 3). In the case of Moneymaker AO silenced lines, we did not notice any modifications in terms of yield, number of fruits per truss or fruit weight under either normal or carbon-limited conditions (Figure 3) in contrast to results in cherry tomato (Garchery et al., 2013). We had hypothesized that in cherry tomato MDHAR silencing led to reduced growth of sink organs (fruits and roots) because they did not receive enough carbon from source organs i.e. photosynthetic leaves but for the moment we are unable to distinguish between the hypotheses that this is because (i) less carbon is available or (ii) the same amount of carbon is available but it is distributed differently between source and sink organs. It also presumably means that under control conditions in Moneymaker carbon is not limiting and no phenotype is seen as the 'source' carbon and/or its transport to fruits is sufficient to overcome the disadvantage of silencing MDHAR. No significant changes in soluble sugar content were observed in leaves or fruit in this study (Supplementary Tables S7 and S8), and although we did not examine early growth in our Moneymaker transgenic lines, we could not detect any differences in vegetative growth of mature plants under optimal conditions (Supplementary table S6). Further hypotheses that could explain the phenotypes involve the mechanisms of hormonal control of fruit set and development in Moneymaker: auxin and gibberellin can regulate fruit set and development (de Jong et al., 2009a; de Jong et al., 2009b) and links between ascorbate metabolism and fruit set have been highlighted (Attolico and De Tullio, 2006; Kotchoni et al., 2009). One possibility is that the ascorbate molecule is necessary as a cofactor during the biosynthesis of gibberellin and auxin (Pastori et al., 2003), its concentration or redox state could therefore alter flowering and fruit set. Another possibility is

that changes occurred in photosynthesis: in our previous work we showed decreased photosynthesis in cherry tomato MDHAR RNAi lines but no significant difference in ascorbate oxidase RNAi lines in cherry tomato. Stomatal conductance also changed, increasing in AO RNAi lines and decreasing in MDHAR RNAi lines (Garchery et al., 2013; Truffault et al., 2016).

### **MDHAR and AO activity and regulation of ascorbate content**

Ascorbate recycling is particularly linked to stress responses and also to growth as summarized by Gallie (2013). The ascorbate biosynthesis pathway is responsible for the accumulation of ascorbate in cells, but over a time-scale of several hours (Bartoli et al., 2006). The improvement of ascorbate recycling by genetic tools has been well studied as efficient recycling might be the key to adapt to sudden changes in the environment. MDHAR is important as the enzyme is localized in multiple cellular compartments (Lisenbee et al., 2005) and its activity allows quick recycling of the radical MDHA into reduced ascorbate. In previous experiments (Gest et al., 2013), we revealed a light-dependent negative correlation between MDHAR activity and ascorbate content in cherry tomato in both leaves and fruits, as also been described by Ren et al (2015). In the present study, we analyzed potential negative control of the ascorbate pool by MDHAR activity in Moneymaker, however we did not observe any significant change in ascorbate content compared to wild type, in contrast to previous results from cherry tomato (West Virginia 106) or Ailsa Craig (Gest et al., 2013; Haroldsen et al., 2011). However, AO activity does seem to be negatively correlated to ascorbate content in this study. DHAR activity often seems to be correlated to ascorbate content in tobacco, maize, potato, tomato and *Arabidopsis* (Chen et al., 2003; Haroldsen et al., 2011; Qin et al., 2011; Wang et al., 2010). MDHAR's influence on ascorbate content still remains unclear as ascorbate content is not always correlated to MDHAR activity in the samples, this might be because MDHA enzymatic metabolism (AO and MDHAR notably, but it should be relevant for APX also) is more likely related to stress tolerance and growth processes than to the regulation of ascorbate content or because of feedback control on the quantities of these metabolites.

### **Fruit MDHAR activity does not affect yield in cherry tomato, in contrast to whole plant MDHAR activity**

Given the link between ascorbate recycling and yield shown in cherry tomato (Truffault et al., 2016), we tested the importance of leaf, or whole plant, ascorbate recycling activities, and

therefore source metabolism, compared to fruit ascorbate recycling activity in the establishment of yield in tomato. Lines under-expressing MDHAR3 at the whole plant level, are characterized by a lower yield than the wild type, which is linked to a reduction in fruit cell size and weight in cherry tomato (Truffault et al., 2016) and confirmed in the present study with *mds5*. The opposite phenotype has been observed for AO RNAi lines (Garchery et al., 2013). One possibility is that hormonal metabolism is perturbed in our transgenic lines, auxin being a good candidate. Ascorbate recycling and oxidation are often related to growth processes (Fry, 1998; González-Reyes et al., 1995; Kato and Esaka, 1999). Using a fruit specific promoter in cherry tomato West Virginia 106, we did not observe any reduction in yield, in contrast to the smaller fruit and lower number of fruits harvested when whole plant activity is modified. Early growth of tomato plantlets was also affected by a reduction in MDHAR activity (Truffault et al., 2016), so it is predominantly sink tissues and organs that are affected. This observation reveals that yield and MDHAR activity in autotrophic tissue (leaves) is more likely to be linked than the activity of sink tissues. Interestingly, auxin biosynthesis has been reported only in vegetative organs and roots (Ljung et al., 2001), but not yet in fruits. In the case of our RNAi lines, a perturbation of leaf MDHAR metabolism may have induced changes in auxin signaling, or metabolism. Another possible link that has been made is the quantity, availability or transport of sugars (photoassimilate) from source to sink organs. An example of a redox enzyme with effects on growth and photochemistry (and therefore leaf chemistry) is DHAR: Chen and Gallie (Chen and Gallie, 2006, 2008) have shown that this enzyme is required for growth and efficient photochemistry, and suggest that DHAR maintains photosynthetic function.

## Conclusion

Moneymaker responds differently to cherry tomato to a reduction in MDHAR or AO activity as effects on yield are not seen under normal conditions or conditions of carbon deficit. Moneymaker therefore seems to be less affected by the changes in the activity of these enzymes than the cherry tomato genotype West Virginia. We have also shown that MDHAR activity in leaf tissue is critical for the establishment of yield as a reduction in fruit MDHAR activity has no impact on yield in cherry tomato. Further studies will be necessary to understand how carbon is accumulated and distributed in these plants following modification of ascorbate recycling enzymes. Genotypic-specific effects on yield stability also point to a source of genetic variability in the response to the ascorbate redox state.

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## Figure legends

### Figure 1

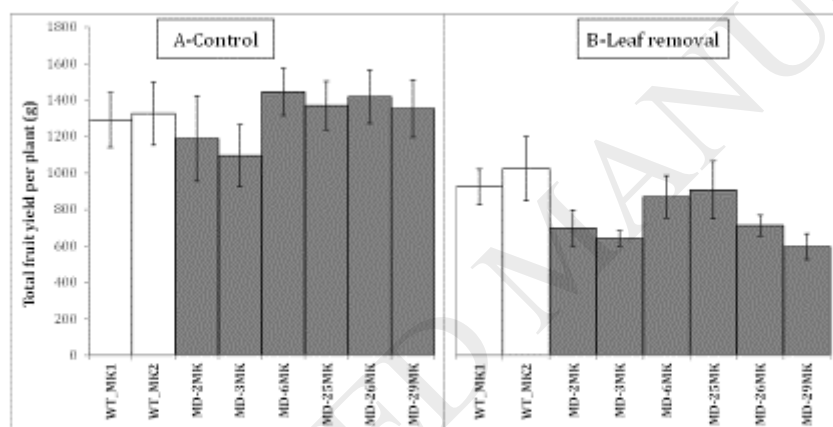
Photos of 70 day-old mature wild-type Moneymaker plants to illustrate a plant grown under conditions of normal culture (left panel) and a plant subjected to carbon stress/carbon limitation by removal of 2 out of three leaves (right panel). Moneymaker plants for the yield experiments were grown in a Venlo-type greenhouse located in Avignon (44°N), France (sowing occurred on the 1<sup>st</sup> August 2012 and planting took place on the 12<sup>th</sup> September 2012). Plants were potted into 5L-pots containing potting compost. Water was supplied to plants using a drip irrigation system to maintain 20-30% drainage. The day/night temperature in the greenhouse was maintained at 22°C/16°C and relative humidity at 70-80%.

Figure 1: Moneymaker plants: control (A) and carbon stress (B)



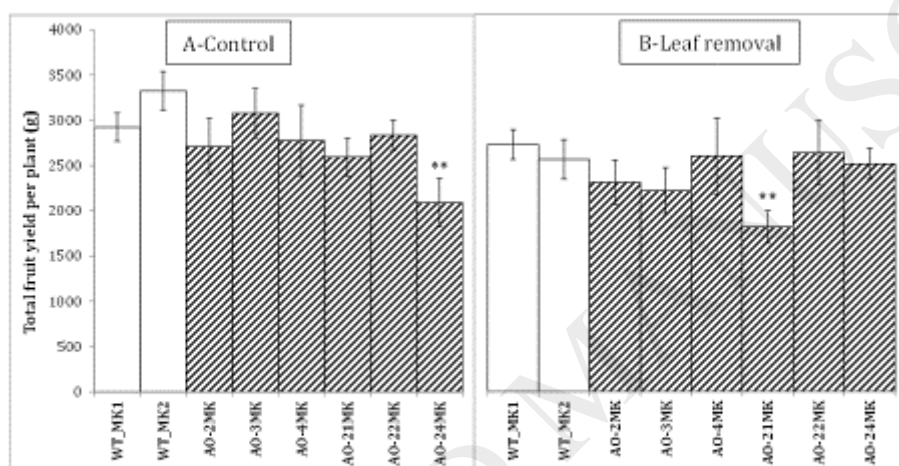
**Figure 2**

Graph showing total fruit yield per plant (in grams) harvested from Moneymaker plants (two wild types and six independent RNAi MDHAR transgenic lines MD-2MK to MD-29MK). Plants were grown in a greenhouse in Autumn under normal growth conditions (A) or carbon-limiting conditions (B). The treatment consisted in removing 2 out of 3 leaves. 4 plants per genotype and per condition were used. Total fruit yield per plant (in grams) was calculated from 6 trusses per plant. Data were analysed together and submitted to a two-way analysis of variance (ANOVA) followed by a Fisher test ( $p < 0.05$ ) but no statistical differences could be noticed between genotype ( $p$ -value=0.622) under each growth conditions. Complete statistical analysis is available in supplementary Table S3 showing a strong treatment effect ( $p$ -value=8.79e-09). Error bars represent standard deviation.

**Figure 2**

**Figure 3**

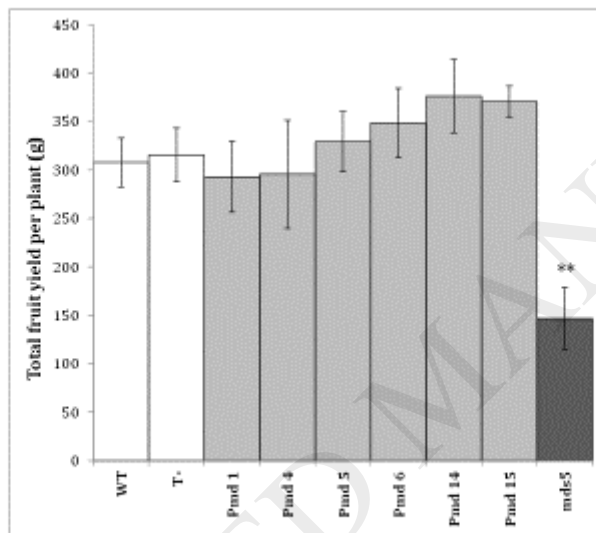
Graph showing total fruit yield per plant (in grams) from 5 trusses per plant harvested from Moneymaker plants (two wild types and six independent RNAi AO transgenic lines AO-2MK to AO-24MK). Plants were grown in a greenhouse in Spring under normal growth conditions (A) or carbon-limiting conditions (B). The treatment consisted in removing 2 out of 3 leaves once fruit were set. 6 plants per genotype and per condition were used. Data were analysed together and submitted to a two-way analysis of variance (ANOVA) followed by a Fisher test ( $p < 0.05$ ). Complete statistical analysis is available in supplementary Table S4 highlighting the treatment effect ( $p$ -value= 0.00513). Error bars represent standard deviation.

**Figure 3**

**Figure 4**

Graph showing total fruit yield per plant (in grams) harvested at maturity from cherry tomato West Virginia 106 MDHAR silenced line (mds5, dark bar), fruit promoter lines (pmds1, pmds4, pmds5, pmds6, pmds14 and pmds15, grey bars) and controls (WT and T-, white bars) grown under optimal growth conditions. At least, six plants per genotype were used. Data were analysed together and submitted to a one-way analysis of variance (ANOVA) followed by a Fisher test ( $p < 0.05$ ). Complete statistical analysis is available in supplementary Table S5. Error bars represent standard deviation.

Figure 4



**Table 1**

MDHAR activity measured in 21-day-old Moneymaker plantlets. Plantlets of wild type (WT\_MK) and MDHAR silenced lines (MD-2MK to MD-29MK) plantlets were grown in a greenhouse in Spring. 6 plantlets per genotype were used as replicates. MDHAR activity is expressed in  $\mu\text{mol NADH oxidized min}^{-1}\text{gFw}^{-1}$ . Different letters indicate different groups as defined by a Tukey test ( $p < 0.05$ ), se represents standard error.

| Line    | MDHAR activity<br>( $\mu\text{mol min}^{-1}\text{g}^{-1}$ ) | se         | p<0.05 |
|---------|---|------------|--------|
| WT_MK   | 0.20  | $\pm 0.04$ | a      |
| MD-2MK  | 0.09  | $\pm 0.02$ | c      |
| MD-3MK  | 0.15  | $\pm 0.04$ | ab     |
| MD-6MK  | 0.08  | $\pm 0.01$ | c      |
| MD-25MK | 0.08  | $\pm 0.02$ | c      |
| MD-26MK | 0.08  | $\pm 0.01$ | c      |
| MD-29MK | 0.10  | $\pm 0.01$ | bc     |

**Table 2**

Ascorbate oxidase activity in 21 days old Moneymaker plantlets. Plantlets of wild type (WT\_MK) and AO silenced lines (AO-2MK to AO-24MK) plantlets were grown in a greenhouse in Spring. 6 plantlets per genotype were used as replicates. AO activity is expressed in  $\mu\text{mol ascorbate oxidized min}^{-1}\text{gFw}^{-1}$ . Different letters indicate different groups as defined by a Tukey test ( $p < 0.05$ ), se represents standard error.

| <b>Line</b> | <b>AO activity<br/>(<math>\mu\text{mol min}^{-1} \text{g}^{-1}</math>)</b> | <b>se</b>  | <b>p</b> |
|-------------|--|------------|----------|
| WT_MK       | 2.23   | $\pm 0.15$ | a        |
| AO-2MK      | 1.87   | $\pm 0.20$ | ab       |
| AO-3MK      | 2.30   | $\pm 0.03$ | a        |
| AO-4MK      | 1.66   | $\pm 0.13$ | b        |
| AO-21MK     | 1.57   | $\pm 0.32$ | b        |
| AO-22MK     | 1.56   | $\pm 0.08$ | b        |
| AO-24MK     | 1.47   | $\pm 0.13$ | b        |



**Table 3**

Average total fruit yield per plant (in grams), fruit number per plant and average fruit weight (in grams) from 6 trusses per plant harvested from West Virginia 106 plants previously described (wild type and two independent RNAi MDHAR transgenic lines). Plants were grown in a greenhouse under normal growth conditions (control) or carbon-limiting conditions (leaf removal). The treatment consisted in removing 2 out of 3 leaves once fruit were set. At least six plants per genotype and per condition were used. Data were analysed together and submitted to a two-way analysis of variance (ANOVA) followed by a Fisher test ( $p < 0.05$ ).

| Treatment    | genotype | Total fruit yield per plant (g) | standard error | Fisher statistical groups |
|--------------|----------|---------------------------------|----------------|---------------------------|
| Control      | WT       | 383.3                           | ±21.0          | a                         |
| Control      | mds3     | 293.8                           | ±27.7          | b                         |
| Control      | mds5     | 300.8                           | ±34.7          | b                         |
| Leaf Removal | WT       | 311.5                           | ±39.0          | ab                        |
| Leaf Removal | mds3     | 259.0                           | ±13.3          | b                         |
| Leaf Removal | mds5     | 155.8                           | ±22.2          | c                         |
| Treatment    | genotype | Number of fruit per plant       | standard error | Fisher statistical groups |
| Control      | WT       | 56.5                            | ±2.9           | ab                        |
| Control      | mds3     | 52.0                            | ±1.9           | bc                        |
| Control      | mds5     | 62.7                            | ±3.0           | a                         |
| Leaf Removal | WT       | 47.3                            | ±4.8           | c                         |
| Leaf Removal | mds3     | 50.5                            | ±1.7           | bc                        |
| Leaf Removal | mds5     | 49.0                            | ±3.1           | bc                        |
| Treatment    | genotype | Average fruit weight (g)        | standard error | Fisher statistical groups |
| Control      | WT       | 6.8                             | ±0.2           | a                         |
| Control      | mds3     | 5.6                             | ±0.3           | bc                        |
| Control      | mds5     | 4.8                             | ±0.5           | c                         |
| Leaf Removal | WT       | 6.6                             | ±0.3           | ab                        |
| Leaf Removal | mds3     | 5.1                             | ±0.2           | c                         |
| Leaf Removal | mds5     | 3.8                             | ±0.2           | d                         |

**Table 4**

Average fruit weight (in grams) from 6 trusses per plant harvested from Moneymaker plants (two wild types and six independent RNAi MDHAR transgenic lines MD-2MK to MD-29MK). Plants were grown in a greenhouse in Autumn under normal growth conditions (control) or carbon-limiting conditions (leaf removal). The treatment consisted in removing 2 out of 3 leaves once fruit were set. 4 plants per genotype and per condition were used. Data were analysed together and submitted to a two-way analysis of variance (ANOVA) followed by a Fisher test ( $p < 0.05$ ).

| Treatment           | genotype | Average fruit weight (g) | standard error | Fisher statistical groups |
|---------------------|----------|--------------------------|----------------|---------------------------|
| <b>Control</b>      | WT_MK1   | 53.7                     | ±5.0           | ab                        |
| <b>Control</b>      | WT_MK2   | 50.8                     | ±1.2           | abc                       |
| <b>Control</b>      | MD-2MK   | 44.3                     | ±7.1           | abc                       |
| <b>Control</b>      | MD-3MK   | 36.9                     | ±5.7           | c                         |
| <b>Control</b>      | MD-6MK   | 37.9                     | ±3.1           | bc                        |
| <b>Control</b>      | MD-25MK  | 45.8                     | ±5.7           | abc                       |
| <b>Control</b>      | MD-26MK  | 38.6                     | ±2.3           | bc                        |
| <b>Control</b>      | MD-29MK  | 48.5                     | ±3.3           | abc                       |
| <b>Leaf Removal</b> | WT_MK1   | 43.6                     | ±7.4           | abc                       |
| <b>Leaf Removal</b> | WT_MK2   | 46.0                     | ±4.6           | abc                       |
| <b>Leaf Removal</b> | MD-2MK   | 46.3                     | ±5.2           | abc                       |
| <b>Leaf Removal</b> | MD-3MK   | 57.4                     | ±11.3          | a                         |
| <b>Leaf Removal</b> | MD-6MK   | 38.1                     | ±5.6           | bc                        |
| <b>Leaf Removal</b> | MD-25MK  | 42.0                     | ±8.9           | abc                       |
| <b>Leaf Removal</b> | MD-26MK  | 35.9                     | ±3.3           | c                         |
| <b>Leaf Removal</b> | MD-29MK  | 46.5                     | ±1.6           | abc                       |

**Table 5**

Number of fruit on 6 trusses per plant harvested from Moneymaker plants (two wild types and six independent RNAi MDHAR transgenic lines MD-2MK to MD-29MK). Data were analyzed as indicated in Table 4.

| Treatment           | genotype | Number of fruits per plant | standard error | Fisher statistical groups |
|---------------------|----------|----------------------------|----------------|---------------------------|
| <b>Control</b>      | WT_MK1   | 24.2                       | ±4.6           | cdef                      |
| <b>Control</b>      | WT_MK2   | 26.2                       | ±5.1           | abcde                     |
| <b>Control</b>      | MD-2MK   | 27.7                       | ±2.9           | abcd                      |
| <b>Control</b>      | MD-3MK   | 29.7                       | ±5.0           | abc                       |
| <b>Control</b>      | MD-6MK   | 38.2                       | ±4.7           | a                         |
| <b>Control</b>      | MD-25MK  | 30.5                       | ±5.0           | abc                       |
| <b>Control</b>      | MD-26MK  | 37.0                       | ±2.5           | ab                        |
| <b>Control</b>      | MD-29MK  | 27.7                       | ±1.4           | abcd                      |
| <b>Leaf Removal</b> | WT_MK1   | 23.0                       | ±2.5           | cdef                      |
| <b>Leaf Removal</b> | WT_MK2   | 23.2                       | ±3.8           | cdef                      |
| <b>Leaf Removal</b> | MD-2MK   | 15.5                       | ±5.9           | def                       |
| <b>Leaf Removal</b> | MD-3MK   | 14.0                       | ±4.6           | ef                        |
| <b>Leaf Removal</b> | MD-6MK   | 24.5                       | ±2.1           | cdef                      |
| <b>Leaf Removal</b> | MD-25MK  | 23.5                       | ±1.8           | cdef                      |
| <b>Leaf Removal</b> | MD-26MK  | 20.2                       | ±3.9           | cdef                      |
| <b>Leaf Removal</b> | MD-29MK  | 12.7                       | ±2.2           | f                         |

**Table 6**

Average fruit weight (in grams) from 6 trusses per plant harvested from Moneymaker plants (two wild types and six independent RNAi AO transgenic lines AO-2MK to AO-24MK). Plants were grown in a greenhouse in Spring under normal growth conditions (Control) or carbon-limiting conditions (Leaf removal). Data were analyzed as indicated in Table 4.

| Treatment           | genotype | Average fruit weight (g) | standard error | Fisher statistical groups |
|---------------------|----------|--------------------------|----------------|---------------------------|
| <b>Control</b>      | WT_MK1   | 73.6                     | ±8.3           | ab                        |
| <b>Control</b>      | WT_MK2   | 76.3                     | ±7.8           | a                         |
| <b>Control</b>      | AO-2MK   | 63.8                     | ±2.6           | abc                       |
| <b>Control</b>      | AO-3MK   | 74.6                     | ±6.9           | ab                        |
| <b>Control</b>      | AO-4MK   | 55.9                     | ±7.8           | abc                       |
| <b>Control</b>      | AO-21MK  | 64.6                     | ±12.8          | abc                       |
| <b>Control</b>      | AO-22MK  | 75.9                     | ±7.1           | a                         |
| <b>Control</b>      | AO-24MK  | 52.0                     | ±1.8           | <b>c</b>                  |
| <b>Leaf Removal</b> | WT_MK1   | 68.4                     | ±4.4           | abc                       |
| <b>Leaf Removal</b> | WT_MK2   | 64.9                     | ±6.0           | abc                       |
| <b>Leaf Removal</b> | AO-2MK   | 60.6                     | ±7.4           | abc                       |
| <b>Leaf Removal</b> | AO-3MK   | 56.0                     | ±2.4           | abc                       |
| <b>Leaf Removal</b> | AO-4MK   | 68.6                     | ±7.4           | abc                       |
| <b>Leaf Removal</b> | AO-21MK  | 55.0                     | ±6.9           | bc                        |
| <b>Leaf Removal</b> | AO-22MK  | 75.9                     | ±9.1           | a                         |
| <b>Leaf Removal</b> | AO-24MK  | 62.7                     | ±8.3           | abc                       |

**Table 7**

Number of fruit on 6 trusses per plant harvested from Moneymaker plants (two wild types and six independent RNAi AO transgenic lines AO-2MK to AO-24MK). Data were analyzed as indicated in Table 4.

| Treatment           | genotype | Number of fruits per plant | standard error | Fisher statistical groups |
|---------------------|----------|----------------------------|----------------|---------------------------|
| <b>Control</b>      | WT_MK1   | 40.8                       | ±3.7           | ab                        |
| <b>Control</b>      | WT_MK2   | 44.5                       | ±3.5           | ab                        |
| <b>Control</b>      | AO-2MK   | 43.5                       | ±5.7           | ab                        |
| <b>Control</b>      | AO-3MK   | 42.6                       | ±5.1           | ab                        |
| <b>Control</b>      | AO-4MK   | 52.7                       | ±8.9           | a                         |
| <b>Control</b>      | AO-21MK  | 41.0                       | ±2.1           | ab                        |
| <b>Control</b>      | AO-22MK  | 38.0                       | ±3.6           | ab                        |
| <b>Control</b>      | AO-24MK  | 40.2                       | ±6.2           | ab                        |
| <b>Leaf Removal</b> | WT_MK1   | 40.2                       | ±3.9           | ab                        |
| <b>Leaf Removal</b> | WT_MK2   | 40.5                       | ±3.1           | ab                        |
| <b>Leaf Removal</b> | AO-2MK   | 41.0                       | ±8.6           | ab                        |
| <b>Leaf Removal</b> | AO-3MK   | 39.3                       | ±5.5           | ab                        |
| <b>Leaf Removal</b> | AO-4MK   | 39.5                       | ±6.4           | ab                        |
| <b>Leaf Removal</b> | AO-21MK  | 32.0                       | ±3.5           | b                         |
| <b>Leaf Removal</b> | AO-22MK  | 36.0                       | ±4.8           | b                         |
| <b>Leaf Removal</b> | AO-24MK  | 42.7                       | ±7.7           | ab                        |