

MANIPULATION OF ASCORBATE
BIOSYNTHESIS IN *Solanum lycopersicum*
(cv. Money Maker)

by

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DECLARATION

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ABSTRACT

Vitamin C (ascorbate or AsA) is a secondary metabolite produced in many eukaryotes including yeasts, plants and animals. It plays essential roles as an anti-oxidant and enzyme cofactor, functions as an electron donor and -acceptor and is involved in various developmental processes. This study was initiated with the aim of increasing vitamin C production in tomato. Three genes, namely GDP-mannose pyrophosphorylase (*GMPase*) from *Saccharomyces cerevisiae*, arabinono-1,4-lactone oxidase (*ALO*) from *Saccharomyces cerevisiae* and *myo*-inositol oxygenase 2 (*MIOX2*) from *Arabidopsis thaliana* were ectopically expressed in the tomato cultivar Money Maker. *GMPase* converts D-mannose-6-P to GDP-D-mannose. This reaction forms part of the well characterized, “Smirnoff-Wheeler” pathway. *ALO* catalyzes the terminal step in erythroascorbate synthesis in yeast. *In situ* it also metabolizes the plant and animal substrates for ascorbate manufacture. *Myo*-inositol (MI) is converted into D-glucuronate by the activity of *MIOX*. D-Glucuronate is a precursor to L-gulono-1,4-lactone synthesis which is the precursor to AsA in animals and thought to be present in plants. The genes were independently introduced with the aid of *Agrobacterium tumefaciens* mediated transformation and expressed under the control of the CaMV 35S promoter. Plants with increased *GMPase* activity consistently showed increased L-ascorbate levels in leaves and fruit of between 20- and 70% compared to the wild-type. Plants transcribing the *ALO* gene exhibited small increases in L-ascorbate in green fruit ($p < 0.1$). Leaf tissue from *MIOX* plants displayed significant activity increases ($p < 0.05$), and substantial decreases in MI. In green fruit two *MIOX* lines had increases in activity, cell wall uronic acids and AsA levels. Marginal increases in L-ascorbate would not warrant industrial application, but follow-up research with over-expression of other enzymes of the “Smirnoff-Wheeler” pathway should be explored.

OPSOMMING

Vitamiën C (askorbiensuur of AsA) is 'n sekondêre metaboliet wat in baie eukariote, insluitend gis, plante en diere geproduseer word. Dit speel 'n noodsaaklike rol as 'n anti-oksidadant en ensiem kofaktor, funksioneer as 'n elektronskenker en aanvaarder en is betrokke in verskillende ontwikkelings prosesse. Hierdie studie was geïnisieer met die doel om vitamien C produksie in tamatie te vermeerder. Drie gene, naamlik GDP-mannose pirofosforilase (GMPase) van *Saccharomyces cerevisiae*, arabinono-1,4-laktoon oksidase (ALO) van *Saccharomyces cerevisiae* en mio-inositol oksigenase 2 (MIOX2) van *Arabidopsis thaliana* was ektopies uitgedruk in die tamatie kultivar, Money Maker. GMPase skakel D-mannose-6-P om na GDP-D-mannose. Hierdie reaksie is deel van die goed gekenmerkde "Smirnof-Wheeler" baan. ALO kataliseer die terminale stap in eritroaskorbiensuur sintese in gis. *In situ* metaboliseer dit ook die plant en dier substrate om askorbiensuur te vervaardig. Mio-inositol (MI) is omgeskakel na D-glukuronsuur deur die aktiwiteit van MIOX. D-glukuronsuur is 'n voorloper in L-guluno-1,4-laktoon sintese wat dan 'n voorloper is van AsA in diere en word ook verdink om in plante teenwoordig te wees. Die gene was onafhanklik ingestel met die hulp van *Agrobacterium tumefaciens* gemedieerde transformasie en uitgedruk onder die beheer van die CaMV 35S promotor. Plante met verhoogde GMPase aktiwiteit het in blare en vrugte konsekwente toename in L-askorbiensuur vlakke met tussen 20 – 70% gewys in vergelyking met wilde-tipe. Plante wat ALO getranskribeer het, het klein stygings in L-askorbiensuur in groen vrugte gewys ($p < 0.1$). Blaarweefsel van MIOX plante wat verhoogde aktiwiteit vertoon het, ($p < 0.05$), het ook aansienlike dalings in MI gehad. In groen vrugte van MIOX het twee lyne verhoogte aktiwiteit, selwand uronsuur en AsA vlakke gehad. Klein toename in L-askorbiensuur is nie gepas vir industriële toepassing nie, maar opvolg navorsing moet ondersoek word met die oor-uitdrukking van ander "Smirnof-Wheeler" baan ensieme.

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List of abbreviations

35S	35S ribosomal subunit
ADP	Adenosine 5'-diphosphate
ALO	Arabinono-1,4-lactone oxidase
AsA	Ascorbic acid
bp	Base pairs
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S promoter
CoA	Coenzyme-A
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
DHA	Dehydroascorbic acid
DTT	1,4-dithiothreitol
EDTA	Ethylenedianinetetraacetic acid
FW	Fresh weight
GMPase	GDP-mannose pyrophosphorylase
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kDa	Kilo Dalton
L-Asc	L-Ascorbic acid
LB	Luria-Bertani
MI	<i>Myo</i> -inositol
MIOX	<i>Myo</i> -inositol oxygenase
mM	Millimolar
MPA	Metaphosphoric acid
MPIMP	Max Planck Institute of Molecular Plant Physiology
mRNA	Messenger ribonucleic acid
MS	Murashige and Schoog
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nopaline synthase terminator
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulphonylfluoride

Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate
PVP-40	Polyvinylpyrrolidone -40
PVPP	Polyvinylpolypyrrolidone
RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
TCA-cycle	Tri-carboxylic acid cycle
TCEP	Tris[2-carboxyethyl]phosphine hydrochloride
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	Catalytic units (μmol product formed per min per mg protein)
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate
WT	Control (wild type)
X-gal	5-bromo-4-chloro-3-indolyl β -D-galactopyranoside

UNDERSTANDING AND INFLUENCING ASCORBATE BIOSYNTHESIS IN
PLANTS

1.1 INTRODUCTION

The evolution of eukaryotic organisms in an oxidative atmosphere has led to a myriad of biochemical adaptations that produce and utilize reactive oxygen species (ROS). The highly reactive ROS are used efficiently in several biochemical processes including oxidation of compounds like glucose, glycolate and xanthine and in the respiratory and photosynthetic electron transport chain. Furthermore, these ROS are crucial as they use their oxidative and signaling abilities in the defense response (Chen et al. 1993; Levine et al. 1994; Foyer et al. 1997). While ROS are produced or utilized in most of the central biochemical pathways of aerobic respiration, accumulation of O_2^- and H_2O_2 can lead to the production of toxic hydroxyl radicals and lipid peroxidases (Bowler et al. 1992). For this reason eukaryotes utilize several antioxidants and antioxidant catabolizing enzymes to prevent the uncontrolled oxidation of mainly membranes, but also other essential cellular components that would result from ROS accumulation within the cell.

In plants, plastidial metabolism and particularly photosynthesis relies on thiol-modulated enzymes such as glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, phosphoribulokinase and sedoheptulose-1,7-bisphosphatase of the Calvin cycle (Kaiser, 1979; Johnson, 2003). These thiol groups are rapidly oxidized by H_2O_2 which must be maintained at a low level. This is achieved through the action of the peroxidase enzymes which utilize a reductant to convert H_2O_2 into H_2O . While numerous reductants have been described, one of the most important for plants is L-ascorbate (vitamin C).

The six carbon lactone, ascorbate (AsA) exists as L-ascorbate and dehydroascorbate (DHA). It is synthesized from D-glucose in plants, yeast and animals, and can comprise up to 10% of the soluble carbohydrate pool (Foyer et al. 1983). AsA is the aldono-1,4-lactone of the hexonic acids, L-galactonic acid (plants) or L-gulonic acid (animals), and is related to the C_6 sugars. It contains an enediol group on C_2 and C_3 and has a C_5 analogue in yeasts and fungi called D-erythroascorbic acid (Davey et al. 2000). The L-enantiomer of AsA is an essential metabolite in plants and animals and is well known for its anti-oxidative properties, integral roles in plant metabolism and close association with photosynthesis and respiration. It is an electron acceptor and -donor and most of its roles stem from its involvement in the cellular redox system.

1.2 ASCORBATE BIOSYNTHESIS

Four proposed pathways of AsA biosynthesis are thought to occur in plants (Figure 1; Appendix: Figure A1). These include, firstly, a pathway that proceeds via D-mannose-1-P (D-Man-1-P) and L-galactose-1-P (L-Gal-1-P) which was coined the "Smirnoff-Wheeler" pathway (Wheeler et al.

1998). A second pathway begins with pectin degradation and uses D-galacturonic acid (D-GalUA) as a precursor (Loewus, 1999; Agius et al. 2003). Two further 'animal-like' pathways use L-gulonono-1,4-lactone (Gull) as terminal substrate. One begins with GDP-D-mannose and proceeds via L-gulose and the other starts with the oxygenation of *myo*-inositol using L-gulonic acid as an intermediate.

AsA biosynthesis in animals proceeds via a different pathway than in plants. Animals convert D-glucose into L-gulonono-1,4-lactone (L-Gull) via the intermediates, D-glucuronate and L-gulonate. L-Gull is then oxidized to AsA by L-gulonono-1,4-lactone dehydrogenase (Lorence et al. 2004).

The Smirnoff-Wheeler Pathway

The best characterized plant AsA pathway is the Smirnoff-Wheeler pathway (Wheeler et al. 1998; Appendix Figure A1). Four of the proteins in this pathway were identified following analyses of a series of vitamin C deficient (*vtc*) *Arabidopsis* mutants. The *de novo* synthesis starts with D-man-1-P which is converted from D-man-6-P by phosphomannomutase. GDP-mannose pyrophosphorylase (GMPase), characterized in the ozone sensitive mutant, *vtc1-1*, then activates it to GDP-D-mannose (GDP-D-Man) (Conklin et al. 1999). GDP-D-Man is epimerized to GDP-L-galactose (GDP-L-Gal) by GDP-D-mannose-3'5'-epimerase (GDPME) (Barber, 1979). In the *vtc2* and *vtc5* mutants, GDP-L-galactose phosphorylase and GDP-L-galactose guanylttransferase proved to be the proteins responsible for the conversion of GDP-L-Gal to L-Gal-1-P (Dowdle et al. 2007; Laing et al. 2007; Linster et al. 2007). L-Galactose (L-Gal) is then synthesized from L-Gal-1-P by the activity of L-Gal-1-P phosphatase (GalPPase) which was first identified in Kiwi fruit and then characterized in the *vtc4-1 Arabidopsis* mutant (Laing et al. 2004; Conklin et al. 2006). Above-mentioned nucleotide sugars are also involved in protein glycosylation and polysaccharide synthesis e.g. rhamnogalacturonan which is a pectin essential for plant development (O'Neill et al. 2004). The production of free L-Gal appears to be a major control point of AsA synthesis as was presented by Loannidi et al. (2009), who showed a strong correlation between GalPPase transcript levels and AsA levels in temperature stressed plants. In the penultimate step, L-Gal is oxidized to L-galactono-1,4-lactone (L-Gall) by NAD-dependent L-galactose dehydrogenase (GalDH). AsA production is finally catalyzed by L-galactono-1,4-lactone dehydrogenase (LGalLDH) located on the inner mitochondrial membrane using cytochrome *c* as electron donor (Bartoli et al. 2000; Wolucka and Van Montagu, 2003 & 2007; Smirnoff et al. 2004).

AsA from the cell wall

In strawberries, L-Gall can also be a product of pectin breakdown to D-galacturonic acid (Loewus, 1999; Agius et al. 2003). L-Galactono-1,4-lactone is produced via the intermediate, L-

galactonate by an aldonolactonase (Agius et al. 2003). This would be a particularly effective carbon recycling system in organs where cell wall breakdown is prevalent, like in ripening fruit. In a recent study, this idea was reinforced by the presence of a very high L-galacturonic acid reductase transcript level in ripe grape berries (Cruz-Rus et al. 2010).

“Animal-like” AsA pathways

Myo-inositol oxygenase (MIOX) catalyzes the ring cleavage of *myo*-inositol to D-glucuronic acid (GlucA), which is an important cell wall precursor (Loewus et al. 1962). Charalampous and Lyras (1957) showed by using radioactive labeling experiments that the *myo*-inositol pathway involves incorporation of carbon into GlucA, L-xylose, L-arabinose and L-gulonic acid, a precursor in the animal AsA biosynthetic pathway. It has been suggested in *A. thaliana* that an animal like AsA pathway exists, starting with *myo*-inositol and hence GlucA as a precursor to AsA (Lorence et al. 2004; Nessler et al. 2006; Zhang et al. 2008).

An alternative *de novo* pathway, involving L-gulose as a product of GDP-D-mannose, terminates in the same substrate as *myo*-inositol, namely L-gulono-1,4-lactone (Wolucka and Van Montagu, 2003). The terminal reaction that occurs in the inner mitochondrial membrane is catalyzed by the cytochrome *c*-dependent, membrane bound proteins, L-galactono-1,4-lactone dehydrogenase and L-gulono-1,4-oxidase (Jain and Nessler, 2000).

AsA recycling

AsA is effectively recycled in the Asada-Halliwel or ascorbate-glutathione (AsA-GSH) cycle (Ishikawa et al. 2006). Ascorbate peroxidase uses AsA as an electron donor to scavenge hydrogen peroxide while AsA is oxidized to monodehydroascorbate (MDHA). MDHA is then either further oxidized to dehydroascorbate (DHA), or reduced back to AsA by MDHA reductase. GSH-dependent DHA reductase can also reduce DHA back to AsA. Glutathione is oxidized in this process and can be reduced back to GSH by NADH-dependent GSH reductase (Davey et al. 2000). Surplus AsA is catabolized into tartrate, threonate and oxalate (Smirnoff and Pallanca, 1996).

The role of AsA in plants

In plants, AsA acts as a free radical scavenger and enzyme co-factor and plays a role in the electron transport chain (Levine, 1986; Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000; Pastori et al. 2003). AsA detoxifies active oxygen species (AOS), e.g. O₂, H₂O₂, O₂⁻ and HO⁻, produced in aerobes. Abiotic stresses such as ozone and UV radiation are largely defended

against by AsA (Conklin et al. 1996). The role of AsA in the defense response extends as far as to regulate transcript levels of certain pathogenesis related (PR) proteins and hormones like abscisic acid (ABA) (Pastori et al. 2003). It participates in cell division, elongation and extension and it is thought to be necessary for fruit ripening (Arrigoni and De Tullio, 2000 & 2002; Green and Fry, 2005). Organic acids like L-oxalic acid, L-glyceric acid, L-tartaric acid and L-threonic acid all require AsA as a substrate (Loewus, 1999; Debolt et al. 2007). An increase in oxidized AsA i.e. DHA, also plays a role by inhibiting the cell cycle (Potters et al. 2000).

1.3 THE ROLE OF ASCORBATE IN HUMANS

With the exception of primates, guinea pigs and Nepalese red-vented bulbuls, mammals synthesize AsA in their livers and birds and reptiles in their kidneys. Those animals not capable of producing AsA have a mutation in the gene that codes for the gulono-1,4-lactone oxidase protein that catalyzes the terminal step in the AsA biosynthetic pathway (Nishikimi et al. 1994).

The role of AsA in the human body

Since humans cannot synthesize or store ascorbate in their own capacity, they rely heavily on plants as a source for this important vitamin. In 1928 AsA was isolated and has since been proven to play roles in the animal and human body as enzyme cofactor, anti-oxidant and in collagen and carnitine biosynthesis (Szent-Gyorgyi 1928; Dunn et al. 1984). AsA serves as an electron donor to eight human enzymes (Wondrack et al. 1978; Stubbe et al. 1985; Englard et al. 1986; Levine et al. 1994). These eight enzymes play roles in collagen hydroxylation (Kivirikko et al. 1985; Peterofsky et al. 1991; Prockop et al. 1995), catecholamine norepinephrine biosynthesis (Kaufman, 1974; Levine, 1991) amidation of peptide hormones (Eipper, 1992; Eipper, 1993), tyrosine metabolism (Lindblad 1970) and carnitine biosynthesis (Dunn et al. 1984; Rebouche et al. 1991). Activity of the above-mentioned enzymes is essential and in their absence, humans are likely to acquire the potentially fatal disease, scurvy (Steward et al. 1953). Because collagen is a structural protein, dental irregularities, hemorrhaging and severe pain in the arms and legs, particularly in the hands and feet, are primary symptoms of scurvy. Signs of scurvy will become evident in men at an intake of less than 10 mg per day (Krebs et al. 1948). Recent research has brought to light novel roles for vitamin C. AsA is proposed to play valuable roles in cardiovascular health by reducing blood pressure, strengthening arterial walls and curbing atherosclerosis (Heitzer et al. 1996). Tveden-Nyborg et al. (2009) showed in guinea pigs that a vitamin C deficiency in the early post-natal period inhibited neuronal development in the hippocampus and that it compromised spatial memory. Large doses of vitamin C have also displayed anti-carcinogenic effects with significant reduction in tumor growth and potential as a prophylactic treatment (Takemura et al. 2010).

The importance of AsA research

AsA production amounts to ~80 thousand tons every year and has an international market value of more than 600 million US\$ (Reviewed by Zhang et al. 2007). It is commercially produced from D-glucose using the Reichstein process which was first patented in 1935 and has been progressively improved (Reichstein, 1935; Hancock and Viola, 2002), but recent advances in AsA biochemistry are steering research into the investigation of alternative AsA production processes. Vitamin C deficiency is rife in impoverished countries where poverty, war and famine have led to severe malnutrition and fruit and vegetables are the main source of vitamin C (Haytowitz, 1995). These communities rely on self-grown crops which can be compromised by abiotic and biotic factors. According to the recommended nutrient intake (RNI) stipulated by the World Health Organisation, Food and Agricultural Organisation of the United Nations, children need 25-40 mg/day (dependant on age) and adults need about 45 mg/day. During pregnancy and lactation, these values increase to 55-77 mg/day. To acquire the RNI for all of the different micronutrients, a rough estimate of 5 fresh fruits and vegetables is advised (Levine, 1986; Prockop and Kivirikko, 1995). The United States Department of Agriculture recommends nutrient intakes of about double the above-mentioned values, although there they encourage the use of excess vitamin C as a prophylactic treatment. In struggling third world countries, this is often an unattainable target if the crops to their disposal are low in vitamin C. Biotechnological approaches are being used extensively to improve the nutritional value of crops through biofortification and to make crops more resistant to abiotic and biotic stresses. Biofortification is by definition the development of crops rich in micronutrients (vitamins and minerals), by using plant breeding and biotechnological methods (Nestel et al. 2006). Traditional plant breeding has been successful to an extent. Orange-flesh sweet potato with high density β -carotene and beans with significantly higher iron levels are a case in point (Van Jaarsveld et al. 2005). There is also the anthocyanin rich P20 blue tomato cultivar that was produced at Oregon State University, the 97L97 tomato cultivar that contains 20 times more β -carotene than regular tomatoes (USDA) and the "Double Rich" tomato cultivar with allegedly 100% increased vitamin C (Growquest; USA). Plant breeding by crossing selected superior traits has its limits though. The plant has to have an established pathway for the synthesis of the required compound. Biotechnology has the potential to fill this gap. An example of successful biofortification through genetic modification is "Golden Rice" which is rich in β -carotene (the precursor to vitamin A) (Ye et al. 2000; Paine et al. 2005). Golden Rice over-expresses phytoene synthase and carotene desaturase, thereby completing the pathway that is usually absent in rice grains. Although not yet commercially available, this cultivar produces enough β -carotene to provide the full RNI to people whose diets consist primarily of rice. Considering that more than one million children die annually because of a vitamin A deficiency, commercial production would make a substantial impact (Zimmerman and Qaim; 2004). Rice has also been engineered for increased iron and zinc (Goto et al. 1999; Ramesh et al. 2004). Tomato

has been engineered in numerous studies, for increases in β -carotene, lycopene and folate (Romer et al. 2000; Ronen et al. 2000; Rosati et al. 2000; Diaz et al. 2007).

1.4 CONTROL OF ASCORBATE BIOSYNTHESIS

Numerous studies have been undertaken with the intent of either further elucidating the ascorbate biosynthetic pathway in plants or to exploit characterized pathways biotechnologically in order to elevate AsA biosynthesis. It is important to identify rate limiting steps in biochemical pathways when attempting ectopic expression studies. There are various factors that can affect the success of such an experiment. Multiple pathways, substrate deficiency, bottle-necking, supply and demand, negative feedback and post-transcriptional silencing are but a few problems that have to be surmounted. Since L-galactose (L-Gal) is used only in AsA synthesis in plants, manipulating it should not have a significant effect on carbon metabolism in the remainder of the plant (Zhang et al. 2007). It is likely for this reason that the Smirnoff-Wheeler pathway is regularly targeted.

Previous attempts at manipulating AsA biosynthesis

GDP-mannose pyrophosphorylase (GMPase) catalyses the first step in the production of all guanosine nucleotide sugars and is mutated in the AsA deficient *vtc 1* mutant (Conklin et al. 1999). Keller et al. (1999) silenced this gene in potato using an antisense construct and found a reduced AsA phenotype and developmental irregularities. Corroborating results were obtained when this gene was expressed in tobacco together with the promoter from the acerola fruit with AsA increases of between 100% and 150% (Badejo et al. 2007).

GDP-L-galactose phosphorylase (*vtc2*) and GDP-L-galactose guanyltransferase (*vtc5*) (Appendix: Figure A1) catalyze the production of L-galactose-1-P. Dramatic increases in AsA of up to 300% were observed in *Arabidopsis thaliana* when GDP-L-galactose transferase from Kiwi fruit was over-expressed, showing the importance of this protein in controlling AsA levels in plants (Bulley et al. 2009).

L-Galactose dehydrogenase (LGalDH) has been the subject of many transformations, but its over-expression or suppression has not been shown to have any significant effect on AsA levels in tomato as was shown by Gatzek et al. (2002) in tobacco. They did however show a decrease in AsA following an *LGalDH* silencing experiment in *Arabidopsis*. Imai et al. (2009) supplemented these data with overexpression and feeding experiments in peach fruit proving that L-Gal levels and NADH are the major determining factors regardless of enzyme activities downstream of L-Gal production.

The galacturonic acid reductase (*GalUR*) gene from strawberry was expressed in *Arabidopsis*, potato and tomato hairy roots with significant increases in AsA (Agius et al. 2003; Hemavathi et al. 2009; Oller et al. 2009). Precursor feeding showed that the pectin breakdown AsA pathway could play a pivotal role if it were not for insufficient substrate.

Stimulation of the terminal enzyme, L-galactono-1,4-lactone dehydrogenase (GLDH) increased AsA levels in sliced potato (Obá et al. 1994) and silencing the GLDH gene resulted in reduced AsA phenotypes in tobacco (Tabata et al. 2001). In strawberry, infiltration with the substrate, L-galactono-1,4-lactone, increased AsA levels without increased GLDH activity (Do Nascimento et al. 2005). In accordance with this, Bartoli et al. (2000, 2005) stated that GLDH activity does not have a significant effect on AsA without the accompanied increase in substrate and the electron acceptor, cytochrome c. (The use of the plant GLDH for the use of anything that increases AsA in any organism has been patented (Smirnoff and Wheeler US20040053235).

Jain and Nessler (2000) achieved a 7-fold increase of AsA in lettuce and tobacco when they ectopically expressed the animal isoform of GLDH, L-Gulono-1,4-lactone oxidase (GLOase) from rat liver. This further promoted the idea of an alternative, animal-like pathway in plants. To exclude the possibility that the GLOase was using the plant terminal substrate, L-galactono-1,4-lactone, the GLOase was expressed in *vtc* mutants and showed elevated ascorbate levels compared to wild-type levels (Radzio et al. 2003).

Lorence et al. (2004) achieved 2-3 fold increases in AsA by over-expressing the *MIOX4* gene in *Arabidopsis thaliana*. Since glucuronic acid is the *myo*-inositol (MI) oxidation product, they hypothesized that a pathway similar to the one found in animals is probably responsible for this increase. Similarly, the increased activity of a novel phytase was proposed to increase AsA levels by increasing free MI by hydrolyzing MI hexakisphosphate, and also following this “animal-like” pathway (Zhang et al. 2008).

The recycling pathway has also been targeted and dehydroascorbate reductase activity increased with a resultant 2 – 4 fold increase in ascorbate levels (Chen et al. 2003).

1.5 PROJECT AIMS AND EXPECTED OUTCOMES

Climacteric fruit like tomatoes, kiwi fruit, melons and bananas are characterized by a concerted and substantial increase in ethylene production and respiration during ripening (Lelièvre et al. 1997). The production of this hormone is modulated autocatalytically, allowing for post-harvest ripening to occur (Oetiker and Yang 1995). It was recently shown in spinach that ethylene levels

decrease AsA synthesis and recycling during senescence (Gergoff et al. 2009). Ethylene also facilitates fruit softening by initiating the breakdown of pectin and other polysaccharides of the cell wall (Balibrea et al. 2000; Nishiyama et al. 2007). During this process, sucrose accumulates as starch is mobilized to provide energy for respiration. All of these factors could influence AsA metabolism in climacteric plants. With many projects focusing on non-climacteric model organisms like *Arabidopsis* and tobacco, tomato was used in this study because it is a good model organism for climacteric fruit metabolism. It is easy to grow, nutritious and of commercial value. The aim of the project was the ectopic expression of yeast (*Saccharomyces cerevisiae*) GDP-mannose pyrophosphorylase (*GMPase*) and arabinono-1,4-lactone oxidase (*ALO*), and *Arabidopsis thaliana* myo-inositol oxygenase genes (*MIOX2*) in the tomato cultivar, “Money Maker” (*Solanum lycopersicum*), and to analyze the effects on ascorbate metabolism.

GMPase expression has only shown increases in AsA when the gene was from acerola. The acerola plant contains exponentially more AsA than most plants (Badejo et al. 2007) and the results suggest that this step in the pathway could be a major control point in AsA biosynthesis.

ALO is an ideal candidate for metabolic engineering of increased AsA because its similarities to the plant isoform (L-galactono-1,4-lactone dehydrogenase) preclude a dependence on cytochrome *c* as an electron acceptor. It can metabolize both L-galactono-1,4-lactone and L-gulonono-1,4-lactone and over-expression in yeast, fed with L-galactono-1,4-lactone, leads to the accumulation of L-ascorbic acid (Hancock et al. 2000; Sauer et al. 2004). This has however only been accomplished in yeast and bacterial hosts with substrate feeding (Lee et al. 1999). In plants, inhibition of Complex I of the respiratory electron transport chain prevents AsA synthesis which occurs in the inner mitochondrial membrane (Millar et al. 2003). Irradiance, ascorbate oxidase activity, cytochrome *c* activity and respiration also play key roles in AsA accumulation (Bartoli et al. 2006; Bulley et al. 2009). These factors can all interfere when trying to manipulate this final step in ascorbate biosynthesis, especially in non-photosynthesizing organs.

With conflicting evidence with regards to the effect of *MIOX* on ascorbate levels, it was expressed in tomato to see if it did indeed increase ascorbate biosynthesis via the proposed glucuronic acid pathway (Lorence et al. 2004; Nessler et al. 2006; Endres and Tenhaken, 2009).

The expected outcome was to observe the translation of an active protein with a flux of carbon into AsA biosynthesis.

ASCORBATE LEVELS IN LEAVES AND FRUITS OF *Solanum lycopersicum* ARE MORE CONSISTENTLY ENHANCED BY ELEVATING GDP-MANNOSE PYROPHOSPHORYLASE ACTIVITY THAN ARABINONO-1,4-LACTONE OXIDASE OR *MYO*-INOSITOL OXYGENASE ACTIVITY

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Abstract

Ascorbate (AsA) plays a fundamental role in redox homeostasis in plants and animals, primarily scavenging reactive oxygen species. With the aim of increasing AsA levels in *Solanum lycopersicum* fruit, three genes, representing distinct steps putatively involved in plant AsA biosynthesis, were cloned and independently expressed under the control of the CaMV 35S promoter. We chose to examine the effect of expressing GDP-mannose pyrophosphorylase (GMPase) and arabinono-1,4-lactone oxidase (ALO) from yeast, as well as myo-inositol oxygenase 2 (MIOX2) from *Arabidopsis thaliana*. Increases in GMPase activity and AsA were concomitant with increased AsA of up to 70% in leaves, 50% in green fruit and 35% in red fruit. However, plants transcribing the ALO and MIOX genes exhibited inconsistent ascorbate levels, indicating that these enzymes do not catalyze crucial steps in AsA biosynthesis and as such do not represent appropriate targets for the manipulation of AsA levels in *S. lycopersicum*. By contrast, GMPase, which catalyzes an early step of the classical “Smirnoff-Wheeler” plant pathway, would appear to exhibit significant control on AsA biosynthesis. Indeed we propose that of the three genes under study, GMPase activity uniquely affects AsA in both the leaves and fruit of *S. lycopersicum*.

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2.1 INTRODUCTION

The L-enantiomer of ascorbate (AsA) or vitamin C acts as a scavenger of the free radicals generated by photosynthesis, cellular respiration and abiotic stresses such as ozone and UV radiation (Levine, 1986; Conklin et al. 1996; Smirnoff and Pallanca, 1996; Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). In addition it has been shown to play roles as an enzyme cofactor while participating in defense, cellular elongation, division and fruit ripening (Arrigoni and De Tullio, 2000, 2002; Pastori et al. 2003; Green and Fry, 2005).

In animals, ascorbate is synthesized from D-glucose which is converted into L-gulono-1,4-lactone (L-GULL) via the intermediates, D-glucuronate (GlucA) and L-gulonate. L-GULL is then oxidized to AsA by L-gulono-1,4-lactone oxidase (Burns and Mosbach, 1956; Figure 1). Humans cannot synthesize ascorbate due to a mutation in the L-gulono-1,4-lactone oxidase gene and therefore have to acquire it through the regular ingestion of fruit and vegetables (Nishikimi et al. 1994). It is the role in collagen biosynthesis that primarily leads to its crucial role in the animal body. Vitamin C deficiency is associated with conditions such as scurvy and low immunity (Reviewed by Padayatty et al. 2003). For this reason attempts to improve the nutritional quality of crops have become a major focus in developing countries where poverty and malnutrition are synonymous (Reviewed by Müller and Krawinkel, 2005). This study was consequently initiated with the aim of increasing vitamin C production in plants, using tomato as a model organism.

The predominant AsA pathway in plants (“Smirnoff-Wheeler” pathway) was initially proposed following the analysis of a series of vitamin C deficient *Arabidopsis thaliana* mutants that accumulated only 30% of the total AsA in wild-type equivalents (Wheeler et al. 1998; Conklin et al. 1999; Conklin et al. 2000). This *de novo* pathway involves the conversion of D-mannose into AsA via a series of L-galactose containing intermediates (Figure 1) (Barber, 1979; Conklin et al. 1999; Bartoli et al. 2000; Wolucka and Van Montagu, 2003 & 2007; Smirnoff et al. 2004; Conklin et al. 2006; Laing et al. 2007; Loannidi et al. 2009). The mitochondrial protein, L-galactono-1,4-lactone dehydrogenase (GLDH), catalyzes the oxidation of L-galactono-1,4-lactone into AsA in the terminal step of this pathway. Alternative pathways to AsA biosynthesis have, however, more recently been proposed. These include a scavenging pathway whereby pectin is degraded into D-galacturonic acid and two other pathways that coincide with the well elucidated animal pathway (Figure 1) viz. a pathway that involves the oxidation of *myo*-inositol (MI) (Lorence et al. 2004; Nessler et al. 2006; Zhang et al. 2008;), and a putative *de novo* pathway where GDP-L-gulose serves as intermediate to L-gulono-1,4-lactone (Wolucka and Van Montagu, 2003).

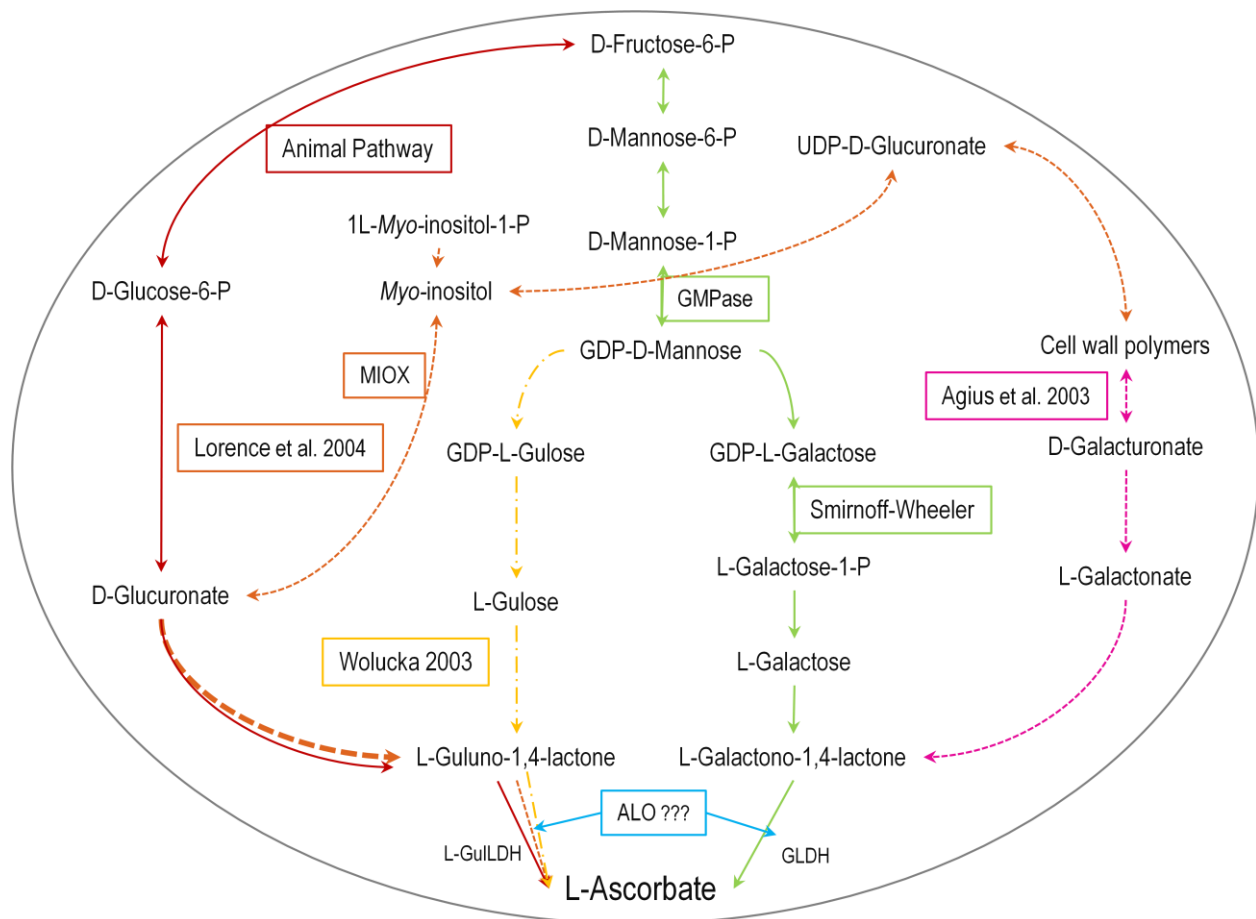


Figure 1: A schematic representation of all the putative pathways towards AsA biosynthesis. The green line is the Smirnov-Wheeler pathway (Wheeler et al. 1998), The pink line is the pectin degradation pathway proposed by Agius et al. (2003). The red line represents the animal AsA biosynthetic pathway with the hypothetical ‘animal-like’ plant pathways in orange (Lorence et al. 2004) and yellow (Wolucka & Van Montagu, 2003). The blue line represents the expected role of the yeast ALO protein in the terminal step of AsA biosynthesis.

The pectin degradation pathway is prominent in strawberry fruit as seen in galacturonic acid reductase (GalUR) over-expression experiments (Loewus, 1999; Agius et al. 2003). High transcript levels of GalUR were also evident in grape berries (Cruz-Rus et al. 2010). Recently, overexpression of a *myo*-inositol oxygenase (*MIOX*) gene in *Arabidopsis* and tobacco increased AsA levels 2-3 fold (Lorence et al. 2004; Nessler et al. 2006). The presence of an “animal-like” pathway was further enforced by a study in which L-gulose also served as an intermediate to the substrate L-gulono-1,4-lactone (Wolucka and Van Montagu, 2003). The terminal reaction for all above-mentioned pathways occurs in the inner mitochondrial membrane and is catalyzed by the cytochrome *c*-dependent, membrane-bound proteins, L-galactono-1,4-lactone dehydrogenase and L-gulono-1,4-oxidase (Jain and Nessler, 2000). In tomato, a recent study in which both

isoforms of GDP-D-mannose 3,5-epimerase (GME) were downregulated provides further evidence for the cell wall-ascorbate nexus (Gilbert et al. 2009). Moreover, temporal analyses of changes in the levels of ascorbate and its precursors (and breakdown products), have indicated that ascorbate metabolism is highly complex in tomato (Carrari et al. 2006; Garcia et al. 2009; Wang et al. 2009).

In an attempt to increase total AsA in tomato, genes putatively associated to the network of AsA biosynthesis were overexpressed in tomato. For this purpose, GDP-mannose pyrophosphorylase (*GMPase*) and arabinono-1,4-lactone oxidase (*ALO*) from *Saccharomyces cerevisiae* and *myo*-inositol oxygenase 2 (*MIOX2*) from *Arabidopsis thaliana* were constitutively expressed in the “Money Maker” cultivar. *GMPase* catalyzes the conversion of D-mannose-1-P to GDP-D-mannose (Conklin et al. 1997). It thus constitutes one of the steps of the “Smirnov-Wheeler” pathway. Silencing of this gene has been demonstrated to result in distinct decreases in ascorbate (Keller et al. 1999), while ectopic expression of the gene has previously been shown to significantly increase AsA (Badejo et al. 2007). *ALO* catalyzes the terminal step in erythroascorbate synthesis in some yeasts, by the oxidation of D-arabinono-1,4-lactone (Huh et al. 1994). This promiscuous enzyme can however also metabolize L-galactono-1,4-lactone and L-gulono-1,4-lactone efficiently in yeast and bacterial hosts, producing AsA (Huh et al. 1994; Lee et al. 1999; Hancock et al. 2000; Sauer et al. 2004; Hancock, 2009). Irradiance, ascorbate oxidase activity, cytochrome *c* activity and respiration play key roles in the rate of the terminal reaction (Millar et al. 2003; Nunes-Nesi et al. 2005; Bartoli et al. 2006; Bulley et al. 2009). By contrast, *ALO* is not sensitive to light or reductant availability in the same way (Bartoli et al. 2006, 2009). *Myo*-inositol is converted into GlucA by the activity of *MIOX*. However the evidence as to whether this GlucA acts as a precursor to AsA in an “animal like” pathway in plants is currently somewhat mixed (Lorence et al. 2004; Zhang et al. 2008, Endres and Tenhaken, 2009).

To recapitulate: *GMPase* has been proven to affect ascorbate biosynthesis in several Solanaceous species (Conklin et al. 1999; Keller et al. 1999; Badejo et al. 2007); *ALO* effectively metabolizes a wide range of substrates towards ascorbate production *in situ* (Huh et al. 1994), and *MIOX* is thought to play a role in an “animal like” pathway. Here we report on the employment of several strategies attempting to increase ascorbate biosynthesis within leaves and fruit of tomato.

2.2 MATERIALS AND METHODS

Constructs and transformations

RNA was extracted from *Arabidopsis thaliana* (L.) Heynh. Ecotype Columbia (Col-O), according to Burgos et al. (1995) with the following modifications. The extraction buffer contained 5% β -mercaptoethanol and RNA was precipitated with one quarter volume 8 M lithium chloride. The dried RNA pellet was reconstituted in ~50 μ L MQ water and RNA concentrations were normalized to 100 ng/ μ L. All samples were DNase treated using DNase I (Fermentas Inc., Burlington, Canada). *Saccharomyces cerevisiae* strain FY23 (S288C) (Winston et al. 1995) inoculum was grown to OD₆₀₀ of 3 – 5 in YPD medium. Cells were harvested by centrifugation (5500 g, 15 m) and resuspended in AE buffer (50 mM Na acetate pH 5.3, 10 mM EDTA). RNA was extracted with phenol/ chloroform at pH 5.3 and precipitated with ethanol. First strand cDNA was produced using 5 μ g DNase-treated RNA from either *S. cerevisiae* or *A. thaliana*. Reactions were performed according to the manufacturer's instructions (Fermentas) using RevertAid™ H Minus Reverse Transcriptase and oligo(dT) primers. The *S. cerevisiae* genes encoding *GMPase* (GenBank accession number NM_001180114) and *ALO* (accession number AY693120.1) as well as the *A. thaliana* *MIOX2* gene (accession number NM_127538) were amplified from the appropriate cDNA using the primers described in Table 1.

The PCR amplification of the genes was performed using *Pfu* polymerase (Fermentas) as per manufacturer's instructions. Each amplified fragment was cloned independently into the pGEM®-T Easy vector (Promega, Madison, WS, USA) according to manufacturer's instructions and transformed into *Escherichia coli* DH5 α . Forward and reverse primers for each of the amplified genes contained an *Xho*I and *Hind*III restriction site respectively. Each fragment was cloned into the corresponding restriction site on the pART7 vector and transferred into the pART27 plant transformation vector as described in Gleave, (1992).

The completed constructs i.e pART27::*GMPase*, pART27::*ALO* and pART27::*MIOX2*, were transformed into *Agrobacterium tumefaciens* EHA 105 cells using the freeze-thaw method as described by Höfgen and Willmitzer (1988). The *Solanum lycopersicum* 'Money maker' cultivar was transformed using *Agrobacterium tumefaciens* mediated transformation as described by Obiadalla-Ali et al. (2004).

Table 1: A list of primers designed for this study (F: forward primer; R: Reverse primer)

#	Name	Bp	Oligo sequence	Intended purpose	Accession no.
1	<i>GMPase F</i>	30	5' GGCTCGAGCATATATAATTGAAAAATGAAAGG '3	Amplify <i>GMPase</i> from cDNA and add <i>XhoI</i> site	NM_001180114
2	<i>GMPase R</i>	29	5' GGAAGCTTAGTTCGTTTTCTAACTCACA '3	Amplify <i>GMPase</i> from cDNA and add <i>HindIII</i> site	
3	<i>ALO F</i>	28	5' GGCTCGAGTCAGGTTTTTACCCCATGT '3	Amplify <i>ALO</i> from cDNA and add <i>XhoI</i> site	AY693120
4	<i>ALO R</i>	30	5' CCAAGCTTACAAAAAGAGACTAGTCGGACA '3	Amplify <i>ALO</i> from cDNA and add <i>HindIII</i> site	
5	<i>MIOX F</i>	29	5' GGCTCGAGTCAAATCCGAGCAAGATGAC '3	Amplify <i>MIOX</i> from cDNA and add <i>XhoI</i> site	NM_127538
6	<i>MIOX R</i>	31	5' GGAAGCTTTGACTCGTAGCTTATCTCACCA '3	Amplify <i>MIOX</i> from cDNA and add <i>HindIII</i> site	
7	<i>GMPase R</i>	21	5' AACAAATGTTGGCACCTGTAGC '3	Sequence specific internal primer for screening and RT-PCR	
8	<i>ALO R</i>	21	5' ATCCCATTGCTTCAAAGGTT '3	Sequence specific internal primer for screening and RT-PCR	
9	<i>MIOX R</i>	20	5' GGGTCGTGCCATTCTCTTA '3	Sequence specific internal primer for screening and RT-PCR	
10	<i>CaMV 35S</i>	21	5' TCCACTGACGTAAGGGATGAC '3	pART27 vector specific FP for screening and RT-PCR	
11	<i>TIP41 F</i>	22	5' ATGGAGTTTTTGAGTCTTCTGC '3	Amplify constitutive gene for RT-PCR	SGN-U321250
12	<i>TIP41 R</i>	19	5' GCTGCGTTTCTGGCTTAGG '3	Amplify constitutive gene for RT-PCR	

Plant material

A stem cutting representing each transformation event was transferred onto MS medium (4.4 g/L Murashige and Skoog, 15 g/L sucrose and 3 g/L agar pH 7) and screened (see below). The stem cuttings transcribing the transgenes of interest were grown in tissue culture for 2 weeks at 22°C under continuous light conditions. Plants were progressively hardened off in the glass house in soil (Double Grow, Durbanville, South Africa) at 22°C in a 16/8 h day night cycle and grown to maturity. Seeds were harvested from ripe fruit of the successfully transformed lines and germinated in the glasshouse (see above). At 4 weeks plants were moved to a greenhouse (summer between months of November and March) under controlled irrigation and supplemented

with 100 mL nutrient solution (50 mL 1g/L calcium nitrate and 50mL 1.5 g/L carbon-free hydroponic nutrient supplement (Hygrotech Hydroponic Nutrients (C-free), Pretoria, South Africa Reg No. K5709) every four days. Leaf samples were harvested at 8 weeks and fruit during green and red stages of maturity (Basson et al. 2010), 25 days and 60 ± 5 days, respectively, post anthesis. All samples were harvested at noon on clear days. In each case, five replicates were sampled for each line, immediately frozen and ground up in liquid nitrogen and stored at -80 °C prior to analysis. The exact same samples were used for all analyses.

Selection of transformants by polymerase chain reaction

Genomic DNA was extracted as described by McGarvey and Kaper (1991) with 0.5g/L spermidine added to the extraction buffer. Approximately 50 mg of tissue was homogenised and incubated at 65 °C and centrifuged (18000 g, 4 °C, 10 min). DNA was extracted from the supernatant with an equal volume of chloroform a total of three times and precipitated with one volume of isopropanol (O/N, 4 °C). GMPase transgenic lines were screened using forward primer 10 and the reverse primer 7 (Table 1). ALO transgenic lines were screened using forward primer 10 and reverse primer 8. MIOX transgenic lines were selected for using the forward primer 10 and reverse primer 9. PCR screening reactions were performed as per manufacturer's instructions using KAPA GoTaq PCR (Kapabiosystems, Woburn, MS, USA) and visualized on a 1% agarose gel containing ethidium bromide (4 µL/ 100 mL). WT plants and plasmids containing the cloned genes of interest were used as negative and positive controls respectively.

RNA extraction and RT- PCR

RNA was extracted from frozen leaf and fruit material according to Burgos et al. (1995) with the following modifications. The extraction buffer contained 5% β-mercaptoethanol and RNA was precipitated with one quarter volume 8 M lithium chloride. The dried RNA pellet was reconstituted in ~50 µL MilliQ water and RNA concentrations were normalized to 100 ng/µL. All samples were DNase treated using DNase I (Fermentas). First strand cDNA synthesis was performed with 5 µg RNA using RevertAid H Minus Reverse Transcriptase (Fermentas). Gene specific forward primers (Table 1, numbers 1-3) and reverse primers (Table 1, numbers 8-10) were used to amplify expressed sequences. *TIP41*, which encodes a TIP41-like protein was used as a constitutively expressed gene control (Table 1 number 11 and 12). Expósito-Rodríguez et al (2008) identified this gene in the Cornell University SOL Genomics Network and found it to be stably expressed in *Solanum lycopersicum* and thus an ideal reference gene for quantitative transcriptomics in this species. All RT-PCR reactions conditions were as follows: 3 min at 94°C; (15 cycles of: 30s at 94°C, 30s at 55°C, 30s at 72°C); 7 min at 72°C.

Protein activity assays

GMPase activity was measured in a total protein extract, from frozen tissue using a radioassay as described by Keller et al. (1999). The assay was started by adding 400 μ L crude protein extract to 400 μ L assay mix (10 mM Tris pH 7.5, 4 mM $MgCl_2$, 5 mM sodium pyrophosphate, 0.1 mM cold GDP-mannose and 0.04 $Cu^{14}C$ GDP-mannose). After 1 h the reaction was stopped with 2 mg activated charcoal and 500 μ L/ 5 mL scintillation fluid measured for radioactivity using the Tri-Carb 2100 TR Liquid Scintillation Analyzer (Packard Instrument Company, Meriden, CT, USA).

MIOX activity was measured using an assay described by Reddy et al. (1981) with the following modifications. Protein was extracted in 10 volumes of ice cold buffer containing 100 mM Tris-HCl pH 7.6, 2 mM L-cysteine, 1 mM ammonium ferrous sulphate hexahydrate, 1 mM EDTA and 1% PVPP. Samples were centrifuged (18000 g, 5 min, 4°C) and one volume 50% PEG 6000 added to the supernatant and the protein precipitated for 30 minutes on ice. Samples were centrifuged (14000 g, 10 min, 4°C) and protein resuspended in 100 mM KPO_4 buffer (pH 7.2) containing 2 mM L-cysteine and 1 mM ammonium ferrous sulphate hexahydrate. MIOX activity was measured in 500 μ g protein, 100 mM KPO_4 (pH 7.2), 2 mM L-cysteine, 1 mM ammonium ferrous sulphate hexahydrate and 60 mM *myo*-inositol. Samples were incubated for 30 minutes at 30°C and boiled for 10 minutes. Denatured protein was removed by centrifugation at (18000 g, 10 min), and glucuronic acid measured as described by Van den Hoogen et al. (1998).

Ascorbic acid measurement

Total ascorbic acid (AsA) was measured as described by Basson et al. (2010), with the following modifications. AsA was extracted in 5 volumes of 6% metaphosphoric acid. L-ascorbate concentration was evaluated against an ascorbic acid standard curve of 0 – 80 μ M. DHA was measured in a 100 mM phosphate-citrate buffer pH 7, containing 70 μ L/ mL plant extract and the concentration calculated against a standard curve of 0-80 μ M DHA. Total ascorbate was measured as the sum of L-ascorbic acid and dehydroascorbic acid.

GC-MS for metabolite profiling

Extraction and derivatization of plant tissue was conducted according to the method of Roessner et al. (2000) with modifications. The polar fraction was extracted from 60 mg frozen leaf tissue homogenized in 1400 μ L 100% methanol and 60 μ L ribitol (0.2mg/ mL water) internal standard. Samples were extracted at 70 °C for 15 m, vortexed and centrifuged (18000 rg, 10 min). The supernatant was added to 1 volume chloroform and 2 volumes water, vortexed and centrifuged (5500 g, 15 min) and the upper phase vacuum dried for derivatization. Dried samples were

reconstituted in 40 μ L methoxyamine hydrochloride (20 mg/ mL in pyridine) and derivatized for 2 hours at 37 °C followed by a treatment (30 min, 37 °C) with 70 μ L MSTFA and 40 μ L internal retention time standard.

Analysis was performed using a 6890N gas chromatograph and 5975 inert mass selective inhibitor mass spectrometer (Agilent Technologies; Santa Clara, CA, USA). Volumes of 1 μ L were injected with a 7683B Series splitless injector (Agilent Technologies;) and gas chromatography was performed on a 30 m Rtx®-5Sil MS Integra Guard column with 0.25 mm internal diameter and 0.25 μ m film thickness (Restek, Bellefonte, PA, USA). Injection- and ion source temperatures were set at 230 °C and 200 °C, respectively, and the program set to 5 m at 70 °C, a first ramp of 1 °C/m to 76 °C and a second ramp of 6 °C/m to 350 °C. The temperature was equilibrated to 70 °C prior to injection of each sample and mass spectra recorded (2 scans per s in range of 50-600 m/z). Data were analyzed using the Enhanced Chem Station MSD Chemstation D.02.00.237 software and compared to the NIST/EPA/NIH Mass Spectral Library (NIST 05) using the NIST Mass Spectral Search Program Version 2.0d.

Preparation of alcohol insoluble residues (AIR) and measurement of cell wall uronic acids

Ethanol was added to ground plant tissue (125 \pm 10 mg) and incubated for 20 m at 70°C. Samples were centrifuged at 8500 g for 10 minutes and supernatants discarded. This process was repeated four times. Samples were washed in acetone, and vacuum dried. Cell wall uronic acids were measured using an adaptation of methods by Blumenkrantz and Asboe-Hansen (1973) and Van den Hoogen et al. (1998). Samples prepared as described above, were weighed (10 mg) and 200 μ L 12 M sulfuric acid added. Samples were vortexed and incubated for 2 h at 4 °C. The sulfuric acid content of the samples was diluted to 2 M and cell wall polysaccharides hydrolyzed for 2 h at 80 °C. Concentrated sulfuric acid containing 120 mM sodium tetraborate was added to 40 μ L aliquots of AIR sample (200 μ L per aliquot) incubated at room temperature for 30 m and background OD measured at 530 nm. Uronic acids were measured as described by Van den Hoogen et al. (1998) against a galacturonic acid standard of 0-8 μ g.

2.3 RESULTS

Constructs, transformations and selection

Stem cuttings of T₀ plants were regenerated in tissue culture and transformants identified by PCR using promoter and gene specific primers 7 – 10 (Table 1). pART27::GMPase, pART27::ALO and pART27::MIOX2 were screened and the results visualized on a 1% agarose gel (Figure 2). Several GMPase lines were positive, but due to an abnormal phenotype, G2 was not further analyzed (Figure 2A). In the case of the ALO transformants, A16 was rejected due to low fruit yield (Figure 2B), however, five other lines were chosen for further analysis. Three of the transformation events with the pART27::MIOX2 construct contained the transgene (Figure 2C).

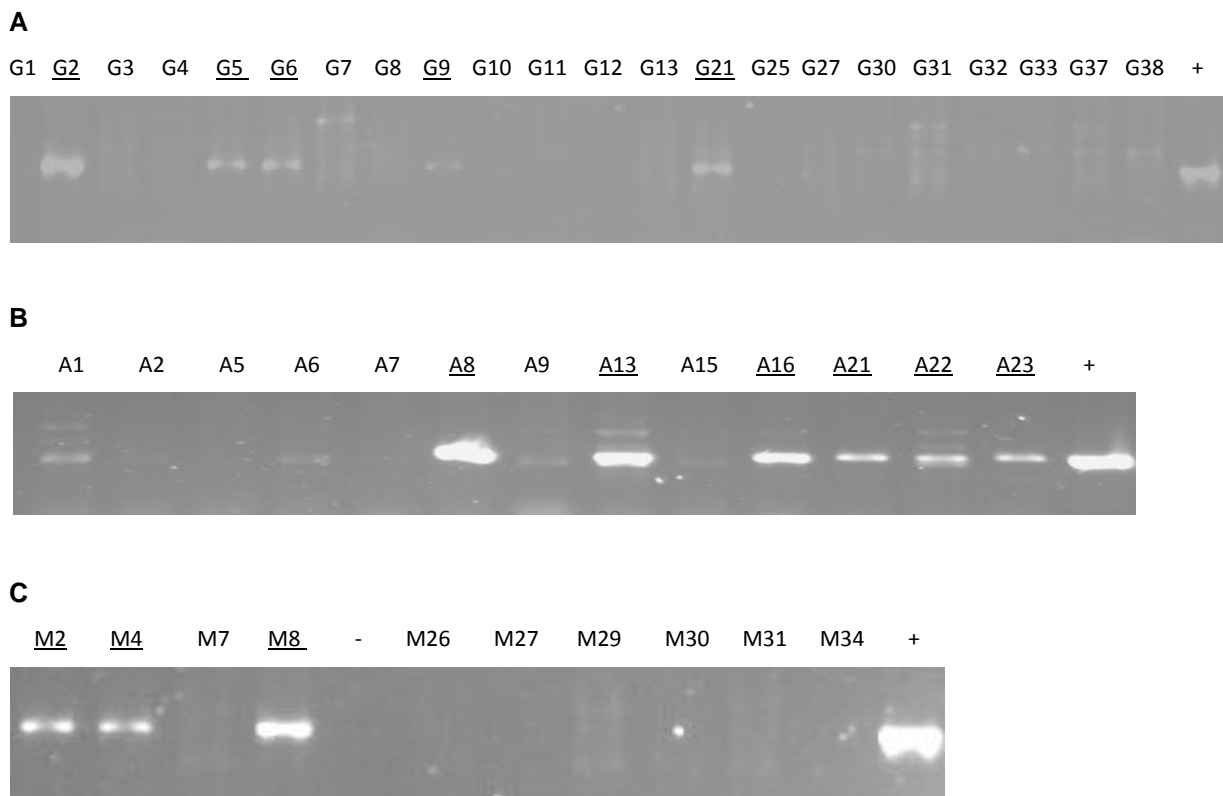


Figure 2: PCR selection of transgenic tomatoes successfully transformed with **A** the GDP-mannose pyrophosphorylase (*GMPase*) gene from *Saccharomyces.cerevisiae* **B** the arabinono-1,4-lactone oxidase (*ALO*) gene from *Saccharomyces cerevisiae*. **C** the *myo*-inositol oxygenase 2 (*MIOX2*) gene from *Arabidopsis thaliana*

GMPase activity

Lines positive for the yeast-derived *GMPase* gene were assayed for activity of the protein using a radiolabel incorporation-assay. Activity was established in tomato leaves and green fruit using the method described by Keller et al. (1999). In comparison to untransformed controls, *GMPase* activity in leaves increased between 26 and 31 times in transgenic lines (Figure 3). In green fruit tissue, activity increased 13 to 17 times.

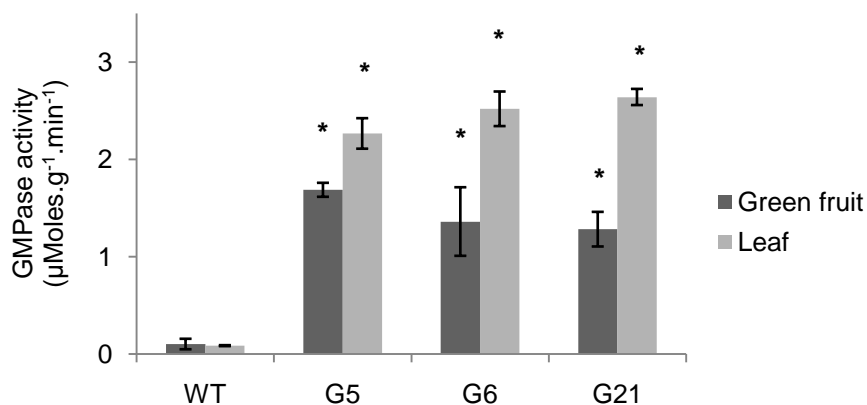


Figure 3: GDP-mannose pyrophosphorylase (*GMPase*) activity measurements in plants expressing *GMPase* from *Saccharomyces cerevisiae*, using [¹⁴C] GDP-mannose, cold GDP-mannose and PP_i as substrates. Activity was measured as the amount of radio label that was incorporated into the product, mannose-1-phosphate. Values calculated as average ± standard deviation; N = 3; p < 0.01

ALO transcription

Arabinono-1,4-lactone oxidase (*ALO*) activity could not be reliably measured because the protein is incorporated into the mitochondrial membrane. Membrane fractions contained varying amounts of active protein, complicating measurement and standardizing of an enzymatic assay. The transcript levels of *ALO* were measured semi-quantitatively and compared to the expression level of the constitutively expressed *TIP41* gene. The nucleic acid bands confirmed the unique transcription of the heterologous gene in transgenic lines (Figure 4A). Following agarose gel separation of RT-PCR products, densitometry measurements of the resulting *ALO* bands relative to the density of each corresponding *TIP41* band revealed similar transcript levels of the *ALO* gene amongst transgenic lines (Figure 4B). Transcription was evident in at least 3 biological replicates of each line.

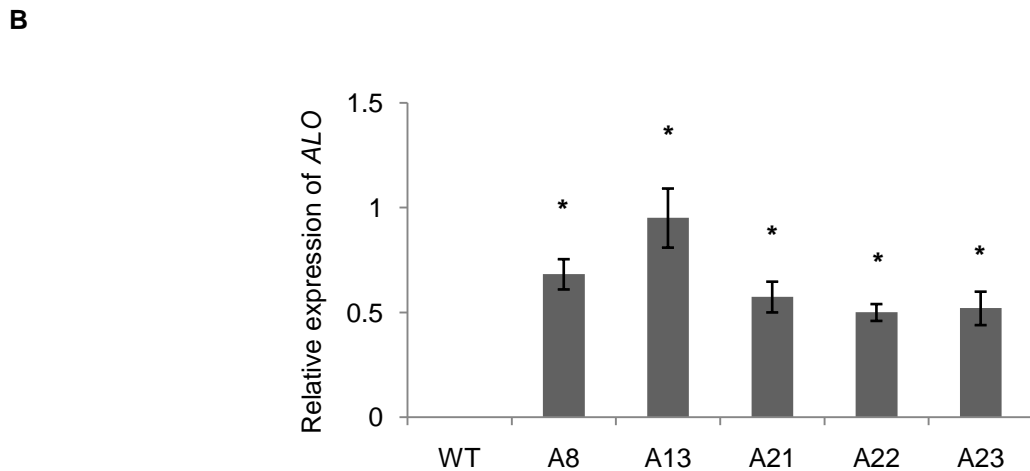
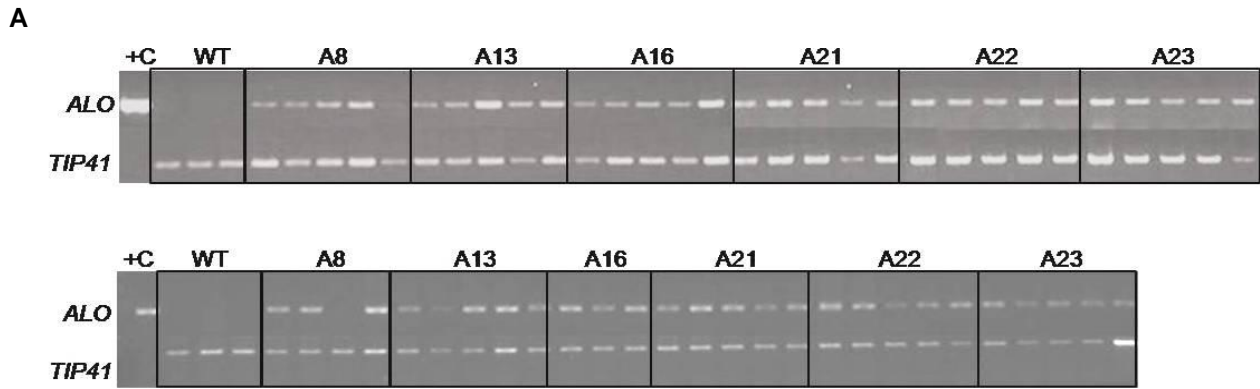


Figure 4: (A) Agarose gel of RT-PCR showing transcription of the arabinono-1,4-lactone oxidase (*ALO*) gene in leaves (top) and green fruit (bottom) of transformed plants using *Tip41* as a constitutively expressed control gene (B) Densitometry analysis of agarose gel depicted in (A). Values were calculated as a ratio between the density of the *ALO* bands and the *Tip41* bands. Values calculated as average \pm standard deviation; N = 3

MIOX activity

An alternative pathway was proposed by Lorence et al. (2004) and Zhang et al. (2008), involving the ring cleavage of *myo*-inositol by *myo*-inositol oxygenase (*MIOX*). *MIOX* activity levels were measured and activity levels were not consistent between leaf, green fruit and red fruit material. All three lines displayed increased *MIOX* activity in the leaves of ~200-300% as compared to wild-type controls. In green fruit, activity in line M8 was not significantly higher than in wild-type plants (Figure 5). Lines M2 and M4 exhibited 2-fold increases at $p < 0.05$ and $p < 0.1$, respectively. By the time the fruit had ripened, there were no significant differences in activity between the *MIOX* lines and wild-type controls (data not shown).

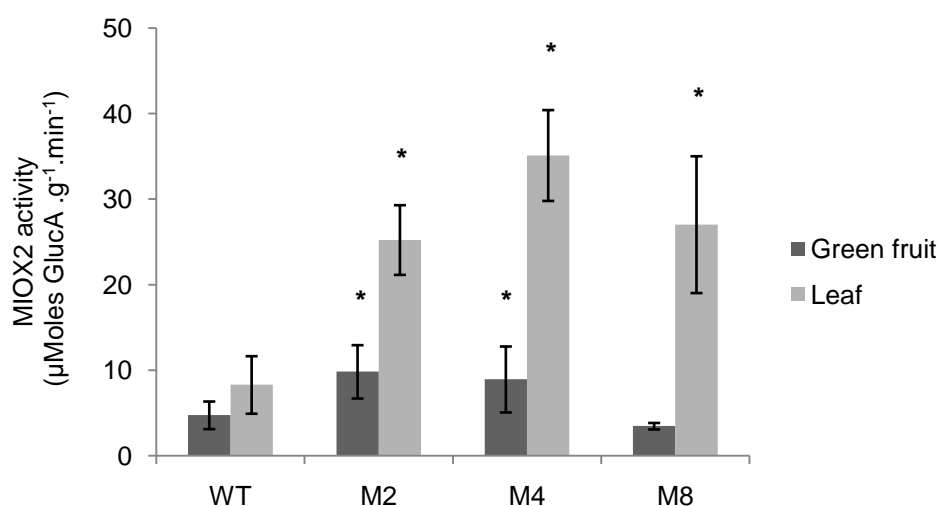


Figure 5: *Myo*-inositol oxygenase (MIOX) activity measurements in leaves of MIOX lines and wild-type controls. *Myo*-inositol was provided as substrate and MIOX activity measured relative to the amount of glucuronic acid produced. This was measured at 540nm before and after samples developed a pink color with addition of a 3-hydroxybiphenylphenol color reagent. Values calculated as average \pm standard deviation; N = 3; $p < 0.1$ (green fruit) $p < 0.05$ (leaves)

Ascorbate

In order to determine whether the introduced transgenes influenced ascorbate biosynthesis or its steady state levels, both L-AsA and DHA were measured. An increase in GMPase activity was positively correlated with ascorbate levels. L-ascorbate content in leaves and green fruit were increased up to 66% and 50% respectively (Table 2) as compared to wild-type controls. L-ascorbate levels were increased to a lesser degree in red fruit, but were still significantly higher (up to 35%) than wild-type red fruit.

Table 2: L-Ascorbate, dehydroascorbate and total ascorbate (AsA) levels measured in leaf, green fruit and red fruit material from plants with increased GDP-mannose pyrophosphorylase (GMPase) activity. Values calculated as average \pm std deviation and measured in μ Moles/g FW. N = 3; *P < 0.1 **P < 0.05

	Leaf			Green fruit			Red fruit
	L-asc	DHA	AsA	L-asc	DHA	AsA	L-asc
WT	1.17 \pm 0.29	0.43 \pm 0.07	1.6 \pm 0.36	0.76 \pm 0.06	0.11 \pm 0.06	0.87 \pm 0.1	0.55 \pm 0.08
G5	1.71 \pm 0.08**	0.53 \pm 0.01**	2.29 \pm 0.07**	1.14 \pm 0.06**	0.13 \pm 0.01	1.27 \pm 0.02**	0.66 \pm 0.03**
G6	1.63 \pm 0.35*	0.5 \pm 0.12*	2.19 \pm 0.46*	1.02 \pm 0.03**	0.11 \pm 0.004	1.13 \pm 0.03*	0.73 \pm 0.02**
G21	1.98 \pm 0.45**	0.64 \pm 0.14*	2.67 \pm 0.59**	1.12 \pm 0.05**	0.16 \pm 0.01	1.28 \pm 0.1*	0.74 \pm 0.11**

L-AsA and DHA were measured in the ALO lines in an attempt to establish a relationship between ALO transcription and AsA levels. AsA was significantly increased ($p < 0.05$) in leaf tissue in all of the lines, except for A13, by between 21% and 54% (Table 3). Transgenic green fruit AsA levels were also significantly increased by up to 25% ($p < 0.1$), in comparison to the wild-type control. That said, the red fruit of the transgenics contained levels of AsA that were invariant from the wild type.

Table 3: L-Ascorbate, dehydroascorbate and total ascorbate (AsA) levels measured in leaf, green fruit and red fruit material from plants transcribing the yeast arabinono-1,4-lactone oxidase (ALO) gene. Values calculated as average \pm std deviation and measured in $\mu\text{Moles/g FW}$. N = 3; *P < 0.1 **P < 0.05

	Leaf			Green fruit			Red fruit
	L-asc	DHA	AsA	L-asc	DHA	AsA	L-asc
WT	1.1 \pm 0.16	0.13 \pm 0.03	1.18 \pm 0.11	1 \pm 0.07	0.16 \pm 0.02	1.12 \pm 0.05	0.97 \pm 0.04
A8	1.2 \pm 0.11	0.17 \pm 0.02*	1.43 \pm 0.16**	1.15 \pm 0.08**	0.2 \pm 0.05*	1.41 \pm 0.01**	0.88 \pm 0.08
A13	1.11 \pm 0.03	0.07 \pm 0.02	1.2 \pm 0.02	1.2 \pm 0.09**	0.27 \pm 0.08**	1.47 \pm 0.01**	1.01 \pm 0.11
A21	1.6 \pm 0.2**	0.17 \pm 0.02*	1.64 \pm 0.19**	1.1 \pm 0.09*	0.27 \pm 0.07**	1.34 \pm 0.04**	0.91 \pm 0.05
A22	1.7 \pm 0.08**	0.15 \pm 0.03	1.82 \pm 0.07**	1.26 \pm 0.1**	0.15 \pm 0.03	1.4 \pm 0.08**	1.02 \pm 0.07
A23	1.52 \pm 0.13**	0.14 \pm 0.12	1.51 \pm 0.07**	1.11 \pm 0.15*	0.25 \pm 0.03**	1.35 \pm 0.19*	0.88 \pm 0.11

The *myo*-inositol oxygenation pathway is proposed to branch after D-glucuronic acid synthesis and feed into AsA biosynthesis (Lorence et al. 2004). Green fruit with increased MIOX activity did indeed display significant increases in AsA levels by 29% and 35% in lines M2 and M4, despite the fact that this pathway is only thought to play a minor role in total ascorbate biosynthesis (Table 4). The line M8 did not exhibit increased activity in the green fruit however; it also did not display increased levels of AsA. Surprisingly, AsA measurements of the leaf tissue contradicted all expected results (Table 4). Samples M2, M4 and M8 had 15%, 30% and 22% lower AsA than the wild-type.

Table 4: L-Ascorbate, dehydroascorbate and total ascorbate (AsA) levels measured in leaf, green fruit and red fruit material from plants containing the *myo*-inositol oxygenase2 (MIOX2) gene. DHA could not be detected in any of the red fruits because the assay is not sensitive enough. Values calculated as average \pm std deviation and measured in $\mu\text{Moles/g FW}$. N = 3; *P < 0.1 **P < 0.05; n/d: not detected

	Leaves			Green Fruit			Red fruit
	L-asc	DHA	AsA	L-asc	DHA	AsA	L-asc
WT	1.61 \pm 0.09	0.09 \pm 0.06	1.65 \pm 0.07	0.65 \pm 0.02	0.03 \pm 0.003	0.69 \pm 0.003	0.55 \pm 0.08
M2	1.34 \pm 0.1**	0.06 \pm 0.02	1.4 \pm 0.1**	0.82 \pm 0.16*	0.07 \pm 0.007**	0.89 \pm 0.06**	0.69 \pm 0.07**
M4	1.2 \pm 0.24**	n/d	1.14 \pm 0.08**	0.9 \pm 0.16**	0.04 \pm 0.008	0.93 \pm 0.08**	0.63 \pm 0.15
M8	1.33 \pm 0.12**	n/d	1.29 \pm 0.07**	0.49 \pm 0.16	0.05 \pm 0.02	0.55 \pm 0.09	0.37 \pm 0.22

Metabolite profiling

In order to determine whether precursor molecules within the various diverse pathways of AsA biosynthesis were affected, GC-MS metabolite profiling was performed on leaf tissue. Upon analysis of the chromatograms and comparison to the NIST library, more than a 150 metabolites were identified in each sample. Several significant deviations were revealed in the metabolite profiles of ALO and MIOX transgenic plants. By contrast, no significant changes in GMPase plants were observed.

Table 5: Metabolite profiling of leaf material from arabinono-1,4-lactone oxidase (ALO) and *myo*-inositol oxygenase (MIOX) lines, together with wild-type controls. GC-MS analysis was used to identify compounds affected by increased ALO and MIOX expression. Values calculated as average \pm standard deviation; N = 3; *p < 0.1 **p < 0.05; n/d: not detected; N/D: not determined

	<i>Myo</i> -inositol	Galactonate	Gluconate	Galactaric acid
WT	10.484 \pm 0.578	0.249 \pm 0.028	-	0.624 \pm 0.024
A8	8.574 \pm 0.279**	0.163 \pm 0.045**	N/D	0.339 \pm 0.008**
A13	10.077 \pm 0.98	0.15711 \pm 0.022**	N/D	0.497 \pm 0.025**
A21	8.942 \pm 0.776**	0.0622 \pm 0.022**	N/D	n/d
A22	8.622 \pm 0.232**	0.199 \pm 0.029	N/D	0.363 \pm 0.099**
A23	9.858 \pm 0.854	0.166 \pm 0.006**	N/D	0.396 \pm 0.022**
WT	10.484 \pm 0.578	0.249 \pm 0.028	0.075 \pm 0.011	0.624 \pm 0.024
M2	n/d**	0.103 \pm 0.041**	0.298 \pm 0.014**	0.208 \pm 0.085**
M4	n/d**	0.097 \pm 0.012**	0.502 \pm 0.129**	0.308 \pm 0.045**
M8	n/d**	0.176 \pm 0.017**	0.303 \pm 0.132*	0.288 \pm 0.005**

Within the metabolite profile of the ALO transgenics, only galactonate was significantly and consistently altered across the lines (changing in A8, A13, A21, A23, but not A22; Table 5). By contrast the *myo*-inositol levels of the MIOX plants were dramatically reduced - even down to undetectable levels (Table 5). These data, however, correspond with previous research reported by Endres and Tenhaken, (2009). Surprisingly, galactonate levels were significantly reduced in all MIOX plants by ~59%, 61% and 29% in M2, M4 and M8 (p < 0.05). In MIOX leaf material, the hexonic acid, gluconate, was significantly increased by 200% to 500% (Table 5). Galactaric acid was significantly decreased (p < 0.05), down to 33% of that observed in the wild-type controls (Table 5).

Cell wall analysis

Cell wall uronic acids were measured in leaf and green fruit tissue of MIOX lines. In the leaf tissue there were small increases in cell wall uronic acids ($p < 0.1$) in all transgenic lines and in green fruit, levels were significantly higher in M2 and M4 by more than 100% ($p < 0.05$) (Figure 6).

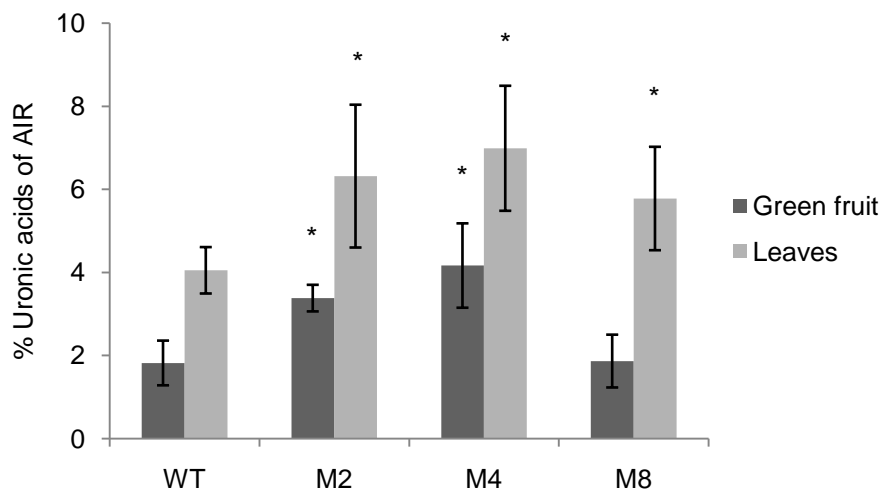


Figure 6: Uronic acid measurements in *myo*-inositol oxygenase (MIOX) lines representative of cell wall biosynthesis. Measurements were conducted on leaf and green fruit material with wild-type controls and expressed as a percentage of total alcohol insoluble residues (AIR) extracted from the cell wall. Values calculated as average \pm standard deviation; $N = 3$; $p < 0.1$ (Leaves); $p < 0.05$ (green fruit)

2.4 DISCUSSION

In order to engineer plants with the ability to accumulate substantially higher levels of ascorbate (AsA), three different approaches were taken in order to (re)direct carbon flux toward AsA biosynthesis. GDP-mannose pyrophosphorylase (*GMPase*), an intermediate constituent enzyme of the Smirnoff-Wheeler pathway, was expressed in an attempt to accelerate the flux of carbon from early to latter stages of the “classical” AsA pathway in order to increase supply of the ultimate precursor of the vitamin. In addition, arabinono-1,4-lactone oxidase (*ALO*), a yeast analogue of the terminal galactono-1,4-lactone dehydrogenase (*GLDH*) gene was expressed in order to assess if increasing the turnover of this terminal step would increase carbon flux towards AsA biosynthesis. The putative plant *myo*-inositol pathway was also considered as a route to this end, and hence *myo*-inositol oxygenase (*MIOX*) was overexpressed. Each of these three genes were ectopically expressed in tomato in an attempt to overcome rate limiting steps in the production of this highly important vitamin or at least to increase the contribution of secondary pathways of its synthesis.

Ectopic expression of GDP-mannose pyrophosphorylase (GMPase)

The transgenic lines G5, G6 and G21 exhibited massively increased *GMPase* activity of between 13- and 27-fold greater than that of the wild type (Figure 3). Even though the wild-type green fruit and leaves had very similar activity levels, all of the transgenic leaf material had significantly more activity than the transgenic green fruit. G5, G6 and G21 leaves had ~30%, ~85% and ~100%, respectively more activity than corresponding green fruit. L-Ascorbate in the *GMPase* transgenic lines significantly increased in leaves, green fruit and red fruit (Table 2). As previously reported, the increases were most apparent in photosynthesizing tissues (Yabuta et al. 2008). On average, leaves showed increased L-ascorbate of approximately 40-70%, green fruit of 30-50% and red fruit of 20-35% ($p < 0.1$). As with the activity levels in these lines, the ascorbate increases were more apparent in leaves. This result is comparable to a previous study, where expression of the *Malpighia glabra* (Acerola) *GMPase* gene under the control of the *Malpighia glabra* *GMPase* specific promoter and the CaMV 35S promoter resulted in AsA increases of about 100% in tobacco leaves (Badejo et al. 2007). It was suggested that this step (*vtc1*), and in particular the two steps prior to L-galactose synthesis denote the major control points in terms of manipulating flux in the direction of AsA production. With such a significant increase in protein activity one would perhaps expect a much more significant increase in AsA. This disproportionate increase is probably due to GDP-D-mannose not only being a precursor for ascorbate but also for GDP-L-fucose, glycoproteins and cell wall polysaccharides (Badejo et al. 2009; Gilbert et al. 2009).

The rate at which AsA is recycled and catabolized can be inferred from the levels of reduced AsA (DHA), glutathione and the breakdown products tartrate and threonate (Pallanca and Smirnoff, 2000). In pea seedlings and in *Arabidopsis* leaves the AsA turnover rate is directly proportionate to the AsA pool size during AsA feeding experiments (Pallanca and Smirnoff, 2000). It was suggested that there is a negative feedback mechanism in place to regulate the AsA pool size. In this study, however, no significant and consistent increases in DHA were apparent (Table 2).

Ectopic expression of arabinono-1,4-lactone oxidase (ALO)

Saccharomyces cerevisiae, D-arabinono-1,4-lactone oxidase (ALO) is a yeast enzyme that has been characterized as a promiscuous protein that catalyzes the rate limiting step in erythroascorbate biosynthesis (Sauer et al. 2004). To our knowledge, ALO has, as yet not successfully been expressed *in planta*. RT-PCR indicated the transcription of the ALO transgene within the ALO lines (Figure 4A and B). AsA levels were significantly higher in leaf tissue of four of the five ALO lines with the level of increase being within 21% - 54% ($p < 0.05$) with respect to the wild-type control (Table 3). Also in green fruit material, AsA levels were significantly higher in all ALO lines by increasing up to 31% ($p < 0.05$; A23: $p < 0.1$) with respect to the wild-type control (Table 3). It is also very interesting to note that the DHA levels in the green fruit were increased far more dramatically in the ALO line than in the GMPase and MIOX lines. This increase in the DHA:L-ascorbate ratio is indicative of increased AsA turnover as a result of an increased AsA pool size (Pallanca and Smirnoff 2000). Even though protein activity could not reliably be established due to technical constraints, it is possible to attribute the small AsA increases in the ALO leaf and green fruit tissues, to the yeast protein. These results partially agree with previous studies which established a correlation between GLDH transcripts and AsA levels (Tamaoki et al. 2003; Ishikawa et al. 2006). Galactonate levels were decreased in transgenic lines according to GC-MS data from leaf tissue (Table 5). Galactonate is the penultimate substrate of the galacturonate pathway and its reduction could conceivably be due to increases in ALO activity. In the lines A8, A21 and A23, there was a correlation between increased AsA levels and decreases in the penultimate precursor of the galacturonate scavenging pathway. By contrast red fruit of the transgenics did not accumulate any more AsA than their respective wild-type controls.

Ectopic expression of myo-inositol oxygenase (MIOX)

Myo-inositol (MI) has been demonstrated in labeling experiments to be incorporated not only into cell wall components, but also into the AsA precursor L-gulonic acid (Lorence et al. 2004; Zhang et al. 2008). Endres and Tenhaken (2009) tried to confirm results suggesting a *myo*-inositol-related AsA pathway by characterizing *MIOX4* overexpressing *Arabidopsis* lines (Endres and Tenhaken, 2009), however, they found these to be largely invariant from wild type. Given these

conflicting results we decided to overexpress the *Arabidopsis MIOX2* gene in transgenic tomato plants.

Transcription of the *MIOX2* transgene was confirmed using promoter and gene specific primers (Table 1; Figure 2C). In leaf material the activity was up to 430% higher in the transgenic plants as compared to the wild-type controls (Figure 5). Similar to results found by Endres and Tenhaken (2009), no increase in AsA was observed in leaf material (Table 4). In fact, a significant decrease in AsA in leaf tissue was actually apparent. Intriguingly this was inversely proportionate to the activity level of MIOX. Given that D-glucuronic acid is a major precursor in cell wall biosynthesis, the levels of uronic acids were measured. When these were expressed as a percentage of the alcohol insoluble residues of the cell wall it was clear that the uronic acid content was significantly higher in MIOX lines than in the wild-type control ($p < 0.1$; Figure 6).

Metabolite profiling was subsequently performed in an attempt to better understand the decrease in AsA in these transgenics. Since *myo*-inositol is directly oxidized by MIOX, and is well represented in GC-MS metabolic profiles, the analysis was performed in order to establish whether there was a decrease in this central molecule in plant metabolism. Steady-state *myo*-inositol levels were decreased to below detectable levels which corroborated the activity data (Table 5; Figure 5). These results therefore imply that MIOX is a rate-limiting enzyme in the *myo*-inositol oxygenation pathway in leaves. Galactonate levels were also significantly reduced in all MIOX plants down to between 80% to 40% of the levels observed in wild type ($p < 0.05$). This observation strongly suggests that there is cross-talk between the presumed animal pathway and the scavenging galacturonic acid pathway (Figure 1). Perhaps the plant is utilizing this scavenging pathway (GalUR pathway), to compensate for the decreasing AsA levels (Table 4). Gluconate is a glucose breakdown product formed by oxidation of the first carbon aldehyde to a carboxyl group. In MIOX leaves, the levels of this hexonic acid increased in M2, M4 and M8 by 200%, 570% and 200% respectively, following the same pattern as the expression and cell wall data. This corresponds with a metabolite study that found a relationship between *myo*-inositol and gluconic acid levels in plants with silenced AsA enzymes (Alhaghdow et al. 2007). The exact reason underlying this event is however currently unclear. Gluconic acid could be an intermediate of AsA catabolism or be connected to glucuronic acid metabolism more closely than is currently thought. Galactaric acid, which is a nitric acid oxidized galactose, was decreased significantly by up to 66% in all MIOX lines compared to wild-type controls ($p < 0.05$) (Table 5). However, currently there is no possibility to explain this decrease.

Green fruit of the lines M2 and M4 maintained their increased MIOX activity, whilst M8 lost its increased expression over time (Figure 5). Interestingly, green fruit of M2 and M4 also had significantly increased AsA levels ($p < 0.05$; Table 4). In order to determine whether increased MIOX activity also had an effect on cell wall biosynthesis, uronic acids were measured in the

alcohol insoluble fractions (Kanter et al. 2005). Significant increases in uronic acids in green fruit were indicative of a shunt of glucuronic acid into the cell wall ($p < 0.05$) (Figure 6). The possibility therefore exists that either carbon is being directed towards AsA biosynthesis through an 'animal-like' pathway or that the increases in cell wall components provide increased substrate for AsA biosynthesis via the pectin breakdown pathway.

In red fruit there was no longer any differential expression of the MIOX protein (results not shown) or an increase in AsA levels. Endogenous *MIOX* transcript levels reduce rapidly as fruit ripens and there is a possibility that the MIOX pathway to AsA biosynthesis is no longer facilitated in mature fruit (Carrari et al. 2006; Loannidi et al. 2009).

2.5 CONCLUSION

Development of biotech crops with enhanced nutritional value and improved agronomic performance has become increasingly popular in parallel to the discovery of a variety of putative ascorbate (AsA) pathways (Hancock 2009). Ectopic expression of selected genes, under the control of a constitutive promoter, provided insight into potential targets to increase vitamin C content in the model fruit-bearing plant tomato. Ectopic expression of GDP-mannose pyrophosphorylase (*GMPase*) in tomato, led to large increases in activity and concomitant increases in AsA. Although results do not imply that this enzyme holds the majority of the metabolic control of this pathway they do provide evidence that increased substrate supply from early steps of the L-galactose pathway can positively affect vitamin C production. Expression of arabinono-1,4-lactone oxidase (*ALO*) lead to less consistent increases in vitamin C suggesting that the native terminal enzyme exhibits relatively little control in unstressed plants in light conditions. *Myo*-inositol oxygenase 2 (*MIOX2*) expression led to increases in AsA in green fruit, but to decreases in the levels of this vitamin in leaves. These results thus suggest that both the terminal step of plant ascorbate biosynthesis and the *Myo*-inositol pathway do not represent effective targets for the manipulation of AsA levels. By contrast, *MIOX* activity and cell wall uronic acid levels strongly correlated suggesting that *MIOX* will likely represent a useful tool for the manipulation of cell wall composition.

The Smirnoff-Wheeler pathway is evidently a more successful target for biotechnological manipulation of ascorbate levels in fruits, with increased *GMPase* activity causing an increased flux towards AsA biosynthesis (Loannidi et al. 2009). For future applications, it would be suggested to express one or more of the genes leading to L-galactose biosynthesis under the control of a fruit-specific promoter.

GENERAL DISCUSSION

In well developed countries, malnutrition is easily curbed by taking multi-vitamin tablets that provide sufficient micronutrients and minerals. Postharvest enrichment is common in nations where food is centrally processed, commercially available and easily attainable. Examples are iodized salt and vitamin D-rich milk. The value of these micronutrient-rich foods is underestimated. In impoverished countries people rely on self-grown staple crops and even when consuming sufficient calories, people in rural communities are still dying from nutrition deficiencies known as MNM (micronutrient malnutrition) (UN).

Genetic engineering, in particular, metabolic engineering, enables researchers to manipulate biochemical pathways by altering endogenous pathways and introducing novel pathways. This gives scientists the platform to progressively and more holistically decipher the complicated and mostly uncharacterized biochemical networks functioning in and between plant cells. Familiar examples are Bt (*Bacillus thuringiensis*) insect resistant crops (<http://www.syngenta.com>), Roundup Ready herbicide tolerant crops (<http://www.monsanto.com>) and “Golden Rice”, which falls within the scope of this project, where biofortification was the primary goal (Ye et al. 2000; Paine et al. 2005). Vitamin C fortification is of massive consequence. In plants it is an essential anti-oxidant, maintaining internal redox homeostasis under abiotic stress conditions and playing an irreplaceable role under biotic stress by activating pathogenesis-related proteins (Pastori et al. 2003). Like with vitamin A and D, folate and zinc, vitamin C is an essential micronutrient in human health, preventing diseases like scurvy.

Poor people consume mostly cereals and starchy storage organs like potato and yams (Food and Agriculture Organization: FAO) that contain a lot of energy, are cheap and easy to grow. The reason for using fruit instead of starchy vegetables in this study is that even though many of them contain some vitamin C uncooked, e.g. potato, they are unpalatable unless cooked. Vitamin C is very labile and gets oxidized at high temperatures, high pH and during storage. In this study, tomato was used as a model for berry fruit and genetically engineered to increase vitamin C production. The ectopic expression of three different genes led to the conclusion that increasing demand at the terminal step did not increase vitamin C in edible fruit. This was also the case when trying to increase flux through a *myo*-inositol oxygenation accessory pathway. The GDP-mannose pyrophosphorylase (*GMPase*) gene proved to be the only one, in this study, that increased vitamin C levels in tomato red fruit. The wild-type Money-Maker tomato cultivar contains approximately 20 mg/100 g (vitamin C/gram of fruit). To obtain 50 mg one would have to consume 250 g of tomato per day. This equates to roughly 2-4* tomatoes (*size depends on growth conditions). With an increase of up to ~33% in the *GMPase* red fruit, one could effectively consume 25% less fruit (188 g) and still receive the same amount of vitamin C. Considering cultivars like the “Double Rich” tomato, (Growquest USA), that contains 100% more vitamin C,

the transgenic lines in this study fall short in terms of commercial or industrial application. This was however just a preliminary study.

Considering the results from this study, attention should be focused primarily on the L-galactose pathway. L-Galactose levels and NADH appear to be the preponderant factors determining ascorbate (AsA) levels in fruit, with the downstream activity of L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase showing little control (Gatzek et al. 2002; Alhagdow et al. 2007; Bulley et al. 2009; Imai et al. 2009). This study suggested that, despite significant increases in AsA, GDP-mannose pyrophosphorylase (GMPase) does not represent a rate limiting step in fruit. In another recent study, the sole over-expression of GDP-mannose-3,5-epimerase (GME) also did not increase AsA levels dramatically (Bulley et al. 2009). GDP-L-galactose guanyltransferase (GGT), GDP-L-galactose phosphorylase (GGP) and L-galactose-1-phosphate phosphatase (GPP) catalyze committed steps in the L-galactose pathway (where GGP and GGT catalyze the same step). GGT from kiwi fruit was over-expressed in tobacco and *Arabidopsis thaliana* and increased AsA by up to 200% and 300% respectively (Laing et al. 2007; Bulley et al. 2009). GPP has not as yet been over-expressed *in planta*, but it has been shown to affect ascorbate and *myo*-inositol metabolism by its ability to hydrolyze L-galactose-1-phosphate as well as D-*myo*-inositol-3-phosphate (Torabinejad et al. 2009). Over-expression of GPP should make for a fascinating study considering the increased interest and research into the proposed *myo*-inositol AsA pathway, complemented with the definitive role of L-galactose levels in the control of AsA levels in fruit.

Since the AsA pathway is affected by a myriad of factors and measurements are difficult due to cell localization, recycling and transport, these have to be understood before the pathway can be effectively exploited. If more than one rate-limiting step exists, it should be attempted to stably transform multiple genes using e.g. a transformation-competent artificial chromosome based system (Lin et al. 2003). In climacteric fruit there is the additional complication of volatile ethylene production. Ethylene affects cell wall metabolism, sucrose levels, and AsA biosynthesis, all of which could influence ascorbate levels in ripening fruit (see Chapter 1.5). Using genetic, proteomic and metabolomic approaches, regulation of transcript levels, post-transcriptional modification and post-translational assembly can be elucidated. With a detailed understanding of the relationship between different AsA pathways and how each one is modulated, L-ascorbate biosynthesis can be manipulated for the production of highly nutritious staple crops and plants with increased tolerance to oxidative stress.

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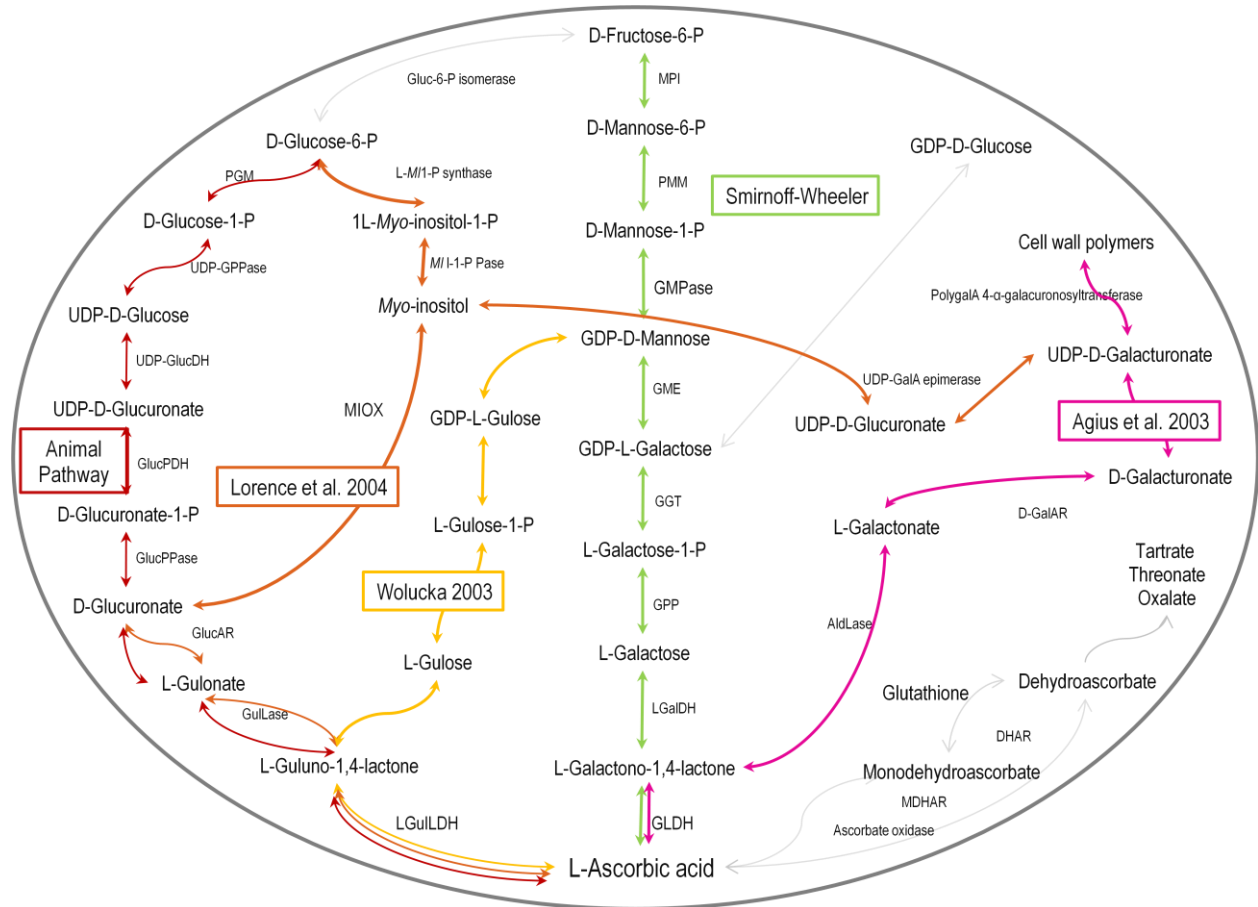


Figure A1: A schematic representation of all the putative pathways towards AsA biosynthesis. The green line is the Smirnov-Wheeler pathway (Wheeler et al. 1998), the pink line is the pectin degradation pathway proposed by Agius et al. (2003). The red line represents the animal AsA biosynthetic pathway with the hypothetical ‘animal-like’ plant pathways in orange (Lorence et al. 2004) and yellow (Wolucka & Van Montagu, 2003). The blue line represents the expected role of the yeast ALO protein in the terminal step of AsA biosynthesis. MPI: Mannose-6-phosphate isomerase; PMM: Phosphomannomutase; GMPase: GDP-mannose pyrophosphorylase; GME: GDP-mannose-3,5-epimerase; GGT: GDP-L-galactose guanyltransferase; GPP: L-Galactose-1-phosphate phosphatase; LGalDH: L -Galactose dehydrogenase; GLDH: L-Galactono-1,4-lactone dehydrogenase; DGalUR: D-Galacturonic acid reductase; Aldase: Aldonolactonase; MDHAR: Monodehydroascorbate reductase; DHAR: Dehydroascorbate reductase; LGuILDH: L-Gulono-1,4-lactone dehydrogenase; GulLase: Gulonolactonase; GlucAR: Glucuronate reductase; GlucPPase: D-Glucuronate-1-phosphate phosphatase; GlucPDH: Glucose-6-phosphate dehydrogenase; UDP-GlucDH: UDP-glucose dehydrogenase; UDP-GPPase: UDP-glucose pyrophosphorylase; PGM: Phosphoglucomutase; MI-1-PPase: *Myo*-inositol-1-P phosphatase; L-MI-P synthase: L-*Myo*-inositol-1-P synthase; MIOX: *Myo*-inositol oxygenase