Project Summary – UA 05/02

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Project title: The role of ascorbate in grape berry metabolism

Executive Summary (Abstract)

Fresh fruits are well accepted as a good source of the dietary antioxidant ascorbic acid (Asc, Vitamin C). However, fruits such as grapes do not accumulate exceptionally high quantities of Asc. Grapes, unlike most other cultivated fruits, use Asc as a precursor for the synthesis of both oxalic (OA) and tartaric (TA) acids. TA is the strongest of the naturally occurring acids in grapes, and its addition to juice, must and wine plays a vital role in controlling the pH. Thus, an understanding of the metabolism of Asc is critical to our understanding of the factors controlling levels of TA in berries during development and at harvest.

Despite the interest in Asc accumulation in many fruits, little is known about the mechanisms by which its cellular concentration is regulated. The purpose of this study was to gain insights into Asc metabolism in wine grapes (*Vitis vinifera* c.v. Shiraz) and thus ascertain whether the developmental demand for TA and OA synthesis influences Asc accumulation. To meet these aims, a method was developed to simultaneously quantify Asc, OA, TA as well as malic acid (MA), which also contributes to the low pH of grape berries. A simple and rapid extraction protocol was established whereby the loss of the labile vitamin C was minimised. Asc and the dicarboxylic acids of interest were separated via Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) and quantified using UV detection. We demonstrated the feasibility of this method to study the distribution of Asc and its catabolites in both fruit and vegetative tissue. This method enabled us to investigate for the first time in grape berries the developmental accumulation of Asc. We were

also able to observe and measure changes to the ratio of the redox couple Asc and dehydroascorbate. These results demonstrate that Asc and its catabolites TA and OA rapidly accumulate during the early stages of grape berry development. The ratio of Asc to DHA increases with berry maturity but fails to reach the high Asc to DHA ratios determined in the leaves. The increase in Asc levels and in the ratio of Asc to DHA observed in ripening grape berries could be the result of a higher Asc biosynthetic capacity, an increased capacity to regenerate Asc via the activity of recycling enzymes or a lower rate of Asc-turnover. To further investigate this, an in-depth developmental analysis of *V. vinifera* c.v's Shiraz and Semillon berries was conducted over two developmental seasons. A combination of metabolite and gene transcription approaches were undertaken to investigate aspects of Asc metabolism including biosynthesis, recycling and catabolism. *V. vinifera* homologous Asc biosynthetic genes of both the primary (Smirnoff-Wheeler) pathway and an alternative 'carbon salvage' pathway were identified. Radiotracer experiments showed that immature berries have a functional Smirnoff-Wheeler Asc biosynthetic pathway operating *in-planta* via the sugar precursors D-mannose and L-galactose. Analysis of the metabolite and gene transcription data demonstrated a positive correlation between the rapid pre-veraison accumulation of Asc in the berries and up-regulation of the Smirnoff-Wheeler Asc biosynthetic genes. Immature berries also accumulated TA in early berry development in co-ordination with up-regulation of a TA biosynthetic gene. In contrast, ripe berries demonstrated up-regulated expression of the alternative 'carbon salvage' pathway gene D-galacturonic acid reductase with only residual expression of Smirnoff-Wheeler pathway genes and of the TA biosynthetic gene L-IdnDN, which encodes the enzyme L-idonate dehydrogenase. The ripening phase was further associated with the following: up-regulation of Asc recycling genes, a secondary rate of increased accumulation of Asc and an increase in the Asc to DHA ratio. We therefore propose that immature berries have a significant demand for TA synthesis, which is met by rapid accumulation of Asc, a low rate of Asc recycling, and a high rate of Asc turnover. We further investigated the influence of the quantity of sunlight on Asc metabolism and thereby on the accumulation of Asc in developing berries. Shaded berries grown in the vineyard were covered with boxes designed to maintain airflow whilst excluding light, and

therefore temperature and humidity were unchanged. Control berries also grown in the vineyard were not shaded and received exposure to sunlight. The influence of bunch shading on the development of the berries, the accumulation of Asc and its catabolites and on the expression of Asc metabolic genes was investigated. The fresh weight of shaded berries was reduced throughout development. Shading significantly reduced the amount of Asc and TA accumulation in developing berries. Shading did not however, have an effect on the developmental accumulation of OA or MA. To investigate whether loss of this environmental stimulus influences transcriptional regulation of Asc metabolic genes, quantitative real-time PCR (QRT-PCR) analysis was conducted. The results demonstrating that bunch shading did not influence expression of Asc-related genes except for one stage of development (28 DAF) where expression of the recycling genes and of one Asc biosynthetic gene was down regulated. These results led us to conclude that whilst prolonged periods of growth of berries without sunlight irradiation of the bunches influences Asc and TA accumulation, light-induced regulation of Asc biosynthetic genes downstream of *GDP-D-mannose-3,5-epimerase* does not occur.

Introduction

The majority of fruit acidity is commonly conferred by malic, citric and ascorbic acids. By comparison, berries of the cultivated grapevine *V.vinifera* predominantly accumulate tartaric acid (TA) and malic acid (MA), which are synthesised during the early phase of berry development. A screen of 78 varieties of *V. vinifera* grapes at two stages of development demonstrated that the ratio of tartaric to malic acid varies between cultivars (*1*). In all cultivars, however, TA is the predominant acid present in the fruit at harvest due to degradation of MA in ripening berries. In the warmer viticultural regions, grapes are often harvested at a later stage of ripeness. These grapes therefore have higher sugar content and lower juice acidity, which can produce unbalanced, flat wines that are more susceptible to oxidative and microbial spoilage (*2*). In these circumstances, grape-derived tartaric acid is added to regulate the pH of the crushed juice, TA being inert to microbial metabolism compared to MA during fermentation. It is clear that MA and TA arise from distinct biosynthetic

metabolic pathways. MA is synthesised from sugars, which are mostly translocated from the leaves (*3*), whilst TA is synthesised from L-(+)-ascorbic acid (*4, 5*). In *V. vinifera* grapes, both oxalic (OA) and tartaric acids are formed by catabolism of ascorbate (*6*).

Ascorbate (Asc) is the most abundant soluble antioxidant found in plant cells, and is present at various concentrations in nearly all fresh food. Since humans have, through evolution, lost the ability to synthesise their own ascorbate, it must be obtained from their diet (*7*). Asc, along with other antioxidants such as flavonoids, polyphenolics and lipophenolics, is often used as an indicator of the nutritional value of foodstuff (*8*). Asc has been the focus of much attention due to the versatility of its cellular functions and its impact on plant growth and development, as reviewed by Smirnoff (*9*), De Gara (*10*) and Noctor (*11*).

Plants make Asc by *de novo*-synthesis pathways (*12*) and from carbon salvage pathways ((*13*) and see review in (*14*)). De novo synthesis of Asc from GDP-D-mannose can occur via two routes, one from GDP-L-galactose and the other from GDP-L-gulose, catalysed by two distinct epimerization reactions of GDP-D-mannose-3,5-epimerase. The reaction via GDP-L-gulose is a newly discovered branch linking the plant non- inversion pathway to the animal-like inversion pathway of Asc biosynthesis, although the activity of only one enzyme has been reported for this pathway (*15*). The L-galactose non-inversion pathway of Asc biosynthesis predominates in green leaves (*12, 16*), and has recently received strong biochemical and molecular support (*17-23*). Molecular support for an alternative route to Asc from pectin-derived D-galacturonic acid was recently provided by the isolation and characterization of NADPH-dependent D-galacturonate reductase from strawberry, which converts D-galacturonic acid to L-galactonic acid This intermediate compound is then readily converted to the final Asc precursor L-galactono-1,4-lactone (*24*). Transgenic *Arbabidopsis* over-expressing strawberry D-galacturonate reductase was shown to accumulate two to three-fold the levels of Asc in wild-type plants (*24*).

Asc concentration varies between species and between tissues of the same species (sees reviews (*25, 26*)). For example, Asc concentration tends to be high in meristematic tissue such as in germinating seedlings (*27, 28*) and in root apex cells (*29*). The biosynthesis of Asc is not the only factor regulating cellular its concentration; Asc is also influenced by external stimuli such as nutrition, as reviewed by Mozafar (*30*), light (*31, 32*) and temperature (*33*).

Light irradiance also influences the Asc pool size, which is not surprising given the role of Asc in photosynthesis (*34*). Leaves grown at high irradiance have a higher Asc content than those grown at low irradiance (see reviews (*35*) and (*36*)). The Asc pool size in some species also decreases when grown in the dark (*36, 37*), although this can be partially reversed by feeding sucrose or glucose (*36*). Recently Gatzek, Wheeler and Smirnoff (*18*) reported that high light acclimation of the antisense L-galactose dehydrogenase *Arabidopsis* plants resulted in increased accumulation of L-galactose. Their report suggests that light-dependent regulation of Asc biosynthesis exists, although the influence of light on turnover is yet to be determined. Although the influence of light on Asc accumulation has not previously been investigated in grape berries, it has been reported that exposure of bunches to high sunlight resulted in maximum accumulation of TA (*38, 39*).

There is also evidence that the expression of some Asc biosynthetic genes is influenced by light. Tabata et al. (*40*) showed that the mRNA of two Asc biosynthetic genes GDP-D-mannose pyrophosphorylase (*GMPase*) and L-galactono-1,4-lactone dehydrogenase (GalLDh) decreased in tobacco leaves grown in the dark and was increased by re-irradiation. However, light-dependent expression of *GalLDh* was not observed in another experiment where shorter acclimation periods in the light and dark were conducted (*41*). Diurnal regulation of GalLDh mRNA expression and activity and of Asc content was demonstrated in *Arabidopsis* leaves. However the diurnal regulation was not observed when plants were grown in the dark suggesting that the Asc pool size is regulated by light rather than the circadian rhythm (*32*).

Asc is not a stable metabolic end-product nor is it limited to oxido-reductase reactions which alter the balance of Asc to DHA. Asc can also be catabolised to oxalic acid, L-threonate and L-tartaric acid in some species as reviewed by DeBolt, Melino and Ford (*42*) and by Banhegyi and Loewus (*43*). In geraneaceous plants, Wagner and Loewus (1973) demonstrated that cleavage of ASC between carbon atoms 2 and 3 results in the formation of OA from carbon atoms 1 and 2, and L-threonic acid (which may be further oxidized to form TA) from carbon atoms 3 to 6. The conversion of DHA to oxalate/L-threonate via the intermediate 4-O-oxalyl-L-threonate was more recently reported (*44*). In *vitaceous* species, cleavage of the Asc catabolic intermediate 5-keto-D-gluconic acid between carbon atoms 4 and 5 leads to TA formation, with the two-carbon fragment of atoms 5 and 6 putatively recycled into central metabolic pathways (*45-47*). Conversion of L-Asc-1-^{14}C to TA in young grapes was first demonstrated by Saito and Kasai (*5*). More recently DeBolt et al (*48*) identified and characterised the enzyme L-idonate dehydrogenase which is responsible for the oxidation of L-idonic acid to 5-keto-D-gluconic acid in the TA biosynthetic pathway. In a pathway distinct from TA biosynthesis, Asc is also cleaved in *vitaceous* species between carbon atoms 2 and 3 leading to OA formation from carbon atoms 1 to 4. A more detailed review of the species differences between Asc catabolic pathways can be found in Loewus (*6*).

Unlike the oxido-reductase reactions which rely on Asc redox enzymes and non-enzymatic reactions to recycle Asc, catabolic reactions require increased Asc biosynthesis to replenish lost Asc. In *Arabidopsis* leaves the loss or turn-over of Asc is only about 2.5% of the pool per hour (*37*) whilst in embryonic axes of pea seedlings, the turn-over is about 13% per hour (*49*). In flowers and early fruits, Asc turn-over was low at 1.41% of the total Asc pool per hour and was increased with fruit maturity to 3% per hour (*49, 50*). However, the rate of Asc turn-over in high oxalate or tartrate accumulators, such as in grapevines is unknown.

The purpose of this research was to investigate Asc accumulation and metabolism in grapevines, which unlike other higher plant species used in similar investigations, is an accumulator of both TA and OA. Genetic, biochemical and metabolite approaches

were taken to study the various facets of Asc metabolism including Asc biosynthesis, Asc recycling and Asc turnover. This research will provide the foundation of our understanding of the genetic, environmental and developmental regulation of Asc metabolism, and thus highlight the targets for future manipulation of the Asc content and subsequent TA and OA levels.

The specific aims of this research are;

1. To develop a rapid extraction, separation and detection method for the simultaneous analysis of Asc/DHA, OA, TA and MA in grapes without compromising the stability of Asc.
2. To determine the distribution of Asc and its catabolites, TA and OA in grapevine tissue and across physiological stages of berry development.
3. To identify full-length sequences of Asc metabolic genes, which share significant homology to those characterised in other high plant species.
4. To develop transcript profiles of Asc biosynthetic, recycling and catabolic genes.
5. To investigate the influence of bunch shading, i.e. light exclusion, on accumulation of Asc, TA and OA.
6. To determine if light-regulation of Asc metabolic genes occurs in grape berries.

Outcomes of this research

1. Method development

- Extraction of total Asc (tAsc), TA, OA and MA was achieved using 3% (w/v) metaphosphoric acid-1 mM EDTA, with minimal exposure of samples to light or to conditions above 4˚C.
- Freeze-thawing of grape extracts tested to ensure this does not disrupt the integrity of Asc and DHA.
- Demonstrated effective recovery of tAsc, TA, OA and MA.

- Reproducible chromatographic separation of each compound was accomplished with detection of Asc and tAsc at 245 nm, and of TA, OA and MA at 210 nm.
- A 'subtraction' method was used for indirect determination of the oxidised form of Asc (DHA) content.
- Application of the method to analyse the distribution of Asc, DHA, TA, OA and MA in photosynthetic and non-photosynthetic tissue and at four stages of physiological development of the berry was demonstrated.

2. Ascorbate metabolism and developmental demand for TA and OA synthesis

- Constructed a metabolite profile for the developmental accumulation of Asc, TA, OA and MA across three seasons in c.v. Shiraz and in two seasons in c.v. Semillon.
- Demonstrated a net rate of increase in the accumulation of Asc, TA and OA across Shiraz berry development
- Asc, TA and OA shown to rapidly accumulate in pre-veraison berries. The rate of Asc accumulation in immature berries is greater than that of TA or OA.
- Seasonal variations in the level of accumulated Asc in developing berries shown to exist.
- Asc levels shown to fluctuate in pre-veraison berries, with a rapid increase followed by a less rapid decrease in accumulated levels.
- Accumulated Asc levels observed to increase in post-veraison berries whilst TA and OA levels are stable during the latter stages of ripening.
- Demonstrated a functional Asc biosynthetic pathway in immature grapes from the precursors D-mannose and L-galactose.
- Demonstrated that rapid recycling of Asc occurs in immature grapes.
- Confirmed that D-mannose, L-galactose and L-ascorbic acid can be metabolized to TA and OA in immature grapes.
- Asc shown to be more rapidly metabolised to TA than D-mannose or L-galactose in the berries; however all precursors are equally effective for the synthesis of TA in the rachis.
- Identification and cloning of full-length grapevine genes with putative Asc biosynthetic functions in the primary Smirnoff-Wheeler Asc biosynthetic

pathway. *GME* encoding GDP-mannose-3,5-epimerase (E.C.5.1.3.18), *vvVtc2* encoding GDP-L-galactose-phosphorylase (EC 2.7.7.12), *vvL-GalDH* encoding L-galactose dehydrogenase (*EC unassigned*) and *vvGaLDh* encoding L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3).

- Identification and cloning of full-length grapevine genes with putative Asc recycling functions *MDHAR* encoding monodehydroascorbate reductase (MDAR EC 1.6.5.4) and *DHAR* encoding dehydroascorbate reductase (DHAR EC 1.8.5.1).
- Demonstrated pre-veraison up-regulation of *vvGME*, *vvVtc2* and *vvL-GalDH* and post-veraison down-regulation of each of these genes.
- Demonstrated that expression of the putative alternate biosynthetic pathway gene *vvGalUR* encoding D-galacturonic acid reductase (E.C. 1.1.1.203) increased with berry ripening.
- Stable expression of *vvGaLDh* shown across berry development.
- Demonstrated that the foliar tAsc content increases from young to expanding stages of maturation, but remains stable in aged leaves. Expression of Asc biosynthetic genes *vvGME* and *vvGaLDh* correlated with the developmental expression in berries.
- Confirmed pre-veraison up-regulation of the TA biosynthetic gene *L-idonate dehydrogenase* with residual expression post-veraison.
- Increasing expression of *MDAR* and *DHAR* with increasing berry and leaf maturation observed.
- The transcript profiles of *MDAR* and *DHAR* shown to correlate with the ripening-associated increase in the ratio of Asc to DHA.

3. The influence of bunch shading on Asc metabolism

- Bunch shading with boxes used successfully to exclude light from the bunch zone without altering the temperature around the berries.
- Chlorophyll content and fresh weight were reduced in bunch shaded berries.
- MA and OA accumulated levels were not influenced by bunch shading.
- Accumulated Asc and TA levels were significantly reduced by bunch shading.

- Expression of a putative Asc biosynthetic gene *vvGalLDh* and of putative recycling genes *vvMDAR* and *vvDHAR* shown to be reduced at only a specific stage (analysis at 28 DAF) of physiological development.
- No other Asc-related genes investigated were influenced by bunch shading.

Discussion and Future Perspectives

The nutritional benefits of Asc are widely recognised by consumers and some fruits are a particularly rich source of dietary Asc. A key function of research in the agrifood sector is the development of nutritionally dense foodstuff (reviewed in (*8*)). This in turn, has encouraged the development of techniques whereby Asc and DHA can be accurately quantified (see review by Davey et al. (*25*)). In addition, interest in the influence of Asc turn-over (via catabolism) on the pool of Asc has sparked renewed interest in TA and OA accumulators particularly as a means of identifying a key target for the *in-planta* regulation or manipulation of Asc content. Our research has resulted in the development of a modified protocol for the extraction of Asc and key organic acids from wine grapes, and the reduction of DHA for total Asc determination. We also present data demonstrating the feasibility of a separation technique that minimised issues with interfering compounds commonly experienced in extractions from plant material. Excellent reproducibility of data values obtained by this HPLC/UV technique was shown, and its application to study the accumulation of these metabolites in vegetative and fruit material, and at various stages of development, is clear. We propose that this method will be feasible for the analysis of these metabolites in other fruits and, furthermore, this method has the potential for high-throughput analysis of multiple samples such as fruit developmental series. Future improvements can be made to the analysis of DHA in fruit extracts. DHA has a weak UV absorption and no response to electrochemical detection and is therefore measured indirectly. We demonstrate that over-estimation of DHA content in fruit extracts can occur with the indirect method, suggesting that the focus must be on developing reliable and direct DHA quantification methods.

Integration of the metabolite, biochemical and transcription data demonstrates a strong developmental regulation of Asc biosynthetic, recycling and catabolic genes in grape berries. Our results indicate that immature berries have a high demand for Asc as a carbon source for large quantities of TA and OA, and to meet this demand Asc biosynthesis must function at a rapid rate. Recently evidence for Asc translocation via the phloem to fruits or tubers has been demonstrated in commercially important crops (*31, 51, 52*), yet the contribution of foliar Asc to the accumulation of Asc in berries is unknown. Radiotracer experiments should be conducted on whole plants to resolve this uncertainty and may indeed provide a further target for manipulating berry Asc content and subsequent TA accumulation. Furthermore, we propose that since DHA contributes to the majority of the Asc pool in pre-veraison berries, which is also the physiological stage of TA synthesis, DHA rather than Asc is a likely candidate as the *in-vivo* precursor for TA. We speculate that the low accumulation of Asc in grape berries is more likely to be a result of a high rate of Asc turnover rather than a low rate of Asc biosynthesis. This is an area that requires further experimental investigation using short-term radiotracer strategies and longer-term genetic engineering techniques using model plant systems and grapevine tools developed to enable rapid transgenic analysis.

The results of our research further indicate that light can influence developmental accumulation of Asc and TA. However, we were unable in this research to identify any Asc-related genes with extended responses to bunch shading. There is evidence in the literature that the expression of some Asc biosynthetic genes is influenced by light. However, the effect of light-exclusion (rather than changes in Asc as a response to stress conditions induced by high light) on Asc accumulation has not previously been studied in fruits, and never been investigated for the entire seasonal growth period. From the outcomes of this research we suggest that light regulation of Asc accumulation occurs in grape berries. Future research should focus on identifying light-responsive genes, which may be involved in mechanisms to regulate Asc accumulation. We suggest that suitable targets could be upstream Asc biosynthetic genes or the active oxygen species scavenger Ascorbate Peroxidase.

In this research we have demonstrated the complexity of factors regulating Asc metabolism in an OA and high TA-accumulator. We propose that developmental, environmental and genetic factors are each involved in determining Asc accumulation in grape berries. The outcomes of our research provide an overview of Asc metabolism in berries, and indicate potential target points for manipulating Asc and TA accumulation in berries. We suggest that whilst Asc metabolism is a viable target for manipulating TA accumulation in berries, careful selection of Asc gene targets is required to avoid interfering with the many other vital functions of Asc. The results of our shading research demonstrate that the reduction in berry TA associated with bunch shading (*39*) reflects reduced accumulation of Asc, the precursor for TA synthesis, under these conditions. Future experiments identifying whether manipulating the quality of light (ratio of far red to red light) rather than the quantity of light will provide a greater understanding of how our current viticulture practices influence the Asc and TA composition of the berry.

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