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Ascorbic Acid Content of Blackcurrant Fruit is Influenced by Both Genetic and Environmental Factors

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ABSTRACT

Blackcurrant (*Ribes nigrum* L.) is a widely grown commercial crop valued for its high ascorbic acid content. In the present study we report large year-to-year variation in the ascorbic acid content of four different blackcurrant cultivars grown at the same site demonstrating strong environmental influence on fruit ascorbic acid concentration. All cultivars examined showed the same trend in ascorbic acid concentration on a year-to-year basis and cultivar hierarchy in fruit ascorbic acid was generally maintained. These data suggest strong underlying genetic determinants for fruit vitamin C concentration and demonstrate that different blackcurrant cultivars show similar responses to prevailing environmental conditions. Linear regression analysis of fruit ascorbic acid content versus several environmental parameters (total solar radiation, total precipitation and average air temperature) suggested a complex environmental interaction with multiple environmental parameters affecting fruit ascorbate. However, pre-harvest solar radiation showed the strongest correlation and data from fruit grown on north or south facing slopes further highlighted the importance of irradiation. Expression analysis of four genes encoding ascorbate biosynthetic enzymes failed to identify any correlations between gene expression and fruit ascorbate content although specific alleles of the gene encoding GDP-D-mannose 3,5-epimerase (E.C. 5.1.3.18) were associated with high fruit ascorbate.

Keywords: GDP-mannose epimerase, GDP-mannose pyrophosphorylase, gene expression, g X e interaction, molecular marker, *Ribes nigrum*, vitamin C
Abbreviations: AsA, L-Ascorbic acid; **QTLs**, quantitative trait loci; **EDTA**, ethylenediaminetetraacetic acid; **GDPMP**, GDP-mannose pyrophosphorylase; **GDPME**, GDP-mannose 3,5-epimerase; **GDPGP**, GDP-L-galactose phosphorylase; **GalPase**, L-galactose-L-phosphate phosphorylase

INTRODUCTION

Blackcurrants are a soft fruit crop widely grown in Europe, New Zealand and to a lesser extent, North America (Brennan 1996). The fruit is widely recognised for its exceptional antioxidant capacity and high concentrations of antioxidant polyphenols, anthocyanins and ascorbic acid compared with other berry fruits (Benvenuti *et al.* 2004). The fruit are primarily used for processing into jams, jellies, nectars and juices and processors are increasingly emphasising the sensory qualities of fruit (colour, flavour) in addition to the potential benefits to health of increased blackcurrant consumption. While many of the antioxidant compounds in blackcurrant fruit represent important sensory compounds with polyphenols providing astringency, modulating mouth-feel (Gawel 2008) and acting as precursors of aroma volatiles (Pichersky and Dudareva 2007) and anthocyanins providing the strong, vibrant colour (Koes *et al.* 2005) their limited bioavailability means they are unlikely to have significant impacts on plasma antioxidant status (Manach *et al.* 2005). In contrast, vitamin C (L-ascorbic acid, AsA) is highly bioavailable (Levine *et al.* 1996) and therefore represents one of the most important antioxidant components in blackcurrant fruit.

Until recently little was known regarding the accumulation of vitamin C in fruit however, in recent years several advances have been made. Biochemical and molecular work has suggested that at least two of the four proposed biosynthetic pathways operate in different fruit with evidence for the salvage pathway from D-galacturonic acid operating in strawberry (Agius *et al.* 2003) and apple (Li *et al.* 2008) and the *de novo* L-galactose pathway operating in blackcurrant (Hancock *et al.* 2007), kiwifruit (Laing *et al.* 2004a, 2004b, 2007), acerola cherry (Badejo *et al.* 2007) and apple (Li *et al.* 2008). In addition to *in situ* synthesis, long-distance phloem transport of AsA from source leaves to fruit represents a potential mechanism for accumulation as has been proposed in apple (Davey *et al.* 2004). A small number of studies have examined expression of biosynthetic genes during fruit development (Agius *et al.* 2003; An *et al.* 2007; Badejo *et al.* 2007) and in the case of acerola, the sequence and structure of upstream promoters have been examined (Badejo *et al.* 2008). In addition, several recent studies have identified quantitative trait loci in apple (Davey *et al.* 2006) and tomato (Rousseaux *et al.* 2005; Stevens *et al.* 2007) contributing towards fruit AsA content. In the case of tomato, for which well established linkage maps are available, two genes encoding enzymes of the L-galactose pathway were found to co-locate with quantitative trait loci (QTLs).

Previous work in blackcurrant demonstrated that AsA accumulation occurred during the early stages of fruit development and that it was dependent on biosynthesis within the fruit via the L-galactose pathway (Hancock *et al.* 2007). However, questions remain regarding the genetic and environmental control over ascorbic acid accumulation in this fruit. Limited genomic resources are available although a number of genetic markers and putative QTLs have been identified for several quality traits that include AsA content (Brennan *et al.* 2008). One purpose of the current study was therefore to investigate the expression and sequence of genes encoding key enzymes of the L-galactose biosynthetic

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pathway in order to gain better insight into the molecular mechanisms controlling AsA accumulation in blackcurrant fruit and to explore the potential use of such sequences as predictive markers for fruit AsA content.

In addition to underlying genetic factors, growing environment is known to play a key role in the accumulation of ascorbic acid in various fruit crops with large variations in ascorbic acid content dependent on growing season (Łata *et al.* 2005; Titinen *et al.* 2005), growing location (Hamner *et al.* 1945; Prior *et al.* 1998) and cultural practices (Caris-Veyrat *et al.* 2004). A second objective of the present study was therefore to determine the impact of growing environment on fruit AsA content and to estimate the underlying genetic stability within different environments.

MATERIALS AND METHODS

Plant material and growth conditions

Plant material was generated and maintained at the Scottish Crop Research Institute, Dundee (56°27′N, 3°04′W). Standard industry regimes for fertilisation and gall mite control were followed. Standard industry fungicide and pest control was applied only during severe outbreaks.

To investigate the impact of growing location on fruit ascorbic acid content, plants of cv. 'Ben Hope' were grown at four separate locations in the UK in the counties of Angus (56°30′N, 3°05′W), Norfolk (52°39′N, 0°50′E), Kent (51°05′N, 0°30′E) and Somerset (51°00′N, 3°11′W). Fruit were collected from 5 whole bushes selected at random at ripeness and three 100 g subsamples were extracted from each bush to give an average AsA value. In Angus, fruit were collected from 5 bushes grown on a North facing slope and 5 bushes grown on a South facing slope independently.

Plant sampling and storage

For ascorbate measurements of genotypes and cultivars, fruit was hand harvested at maturity from a total of 5 plants (3–5 years old). Samples were carefully mixed and subsamples consisting of 150 g fruit frozen at –20°C.

For RNA isolation, leaves or fruit were sampled from plants of different genotypes directly into liquid nitrogen. Tissues were stored at –80°C for up to three months prior to extraction. Fruit stages sampled were closed and open flowers, small green fruit < 6 mm (stage 1), large green fruit > 6 mm (stage 2), green-red fruit (stage 3), red-green fruit (stage 4), red fruit (stage 5) and ripe fruit (stage 6).

Ascorbic acid extraction and quantification

Three replicate samples were removed from frozen storage and allowed to thaw for 4 h. A 150 µl aliquot of Pectinex® 5X (Novozymes A/S, Bagsvaerd, Denmark) was added and fruit was blended in a Waring blender for 60s. Samples were left to digest at room temperature (20°C) overnight and insoluble material removed by centrifugation (5000 × *g*, 1°C, 20 min) followed by filtration (Whatman number 1, Whatman International, Maidstone, UK). The supernatant consisting of the juice fraction was used for ascorbic acid quantification as outlined below and previous work has shown good ascorbate recoveries using this methodology (Walker *et al.* 2006). Between 1972 and 1993, ascorbic acid was quantified by titration against the redox sensitive dye 2,6-dichlorophenolindophenol (DCPIP) based on the method described by Hoffman *et al.* (1970). In brief, samples (1-5 ml) were acidified with 1 ml 1 M HCl and then brought to a final volume of 20 ml with 2% oxalic acid. The solution was then titrated against a 3.3 mM solution of DCPIP and the ascorbic acid content calculated against standard solutions. From 1994, ascorbic acid was quantified by reverse phase HPLC with UV detection at 245 nm. Blackcurrant juice was diluted 1:20 into 5% metaphosphoric acid and centrifuged (16,000 × *g*, 5 min, 1°C) prior to filtration through a 0.2 µm filter. 20 µl of sample were injected onto a 250 × 4.8 mm C18 column and eluted with 30 mM potassium phosphate buffer pH 2.8 at a flow rate of 1.0 ml min^{-1}. Ascorbic acid concentration was determined following integration of the peak area and comparison against a standard curve.

Generation of blackcurrant fruit cDNA library

A cDNA library was constructed in the λZAPII vector system (Stratagene, CA, USA) using mRNA isolated from green/red Ben Alder fruit (Woodhead *et al*. 1998). The cDNA library contained approximately 6.6 x 10^6 primary clones with an average insert size of 900 bp. Following *in vivo* excision of the pBluescript phagemid from λZAPII, colonies containing cDNA inserts were identified and picked into 384-well plates. The phagemids (4 × 384) were single pass sequenced using BigDye® version 3.1 (Applied Biosystems, CA, USA) and the universal M13 reverse primer (5′-CAGGAAACAGCTATGAC). Sequences were analysed using the ABI3730 automated sequencer (Applied Biosystems). From a total of 1536 cDNAs sequenced (4 x 384), 758 sequences were singletons and there were 155 contigs.

RNA extraction and cDNA generation

RNA was extracted from leaves or fruit essentially as described by Iandolino *et al.* (2004) with an additional step for carbohydrate removal (Reid *et al.* 2006). Briefly, samples were ground to a powder in liquid N_2 and transferred to pre-warmed (65°C) extraction buffer consisting of 300 mM Tris-HCl pH 8.0, 25 mM EDTA, 2% (w/v) cetyltrimethylammonium bromide, 2 M NaCl, 2% (w/v) polyvinylpolypyrrolidone, 0.5 g l^{-1} spermidine and 2% (v/v) 2-mercaptoethanol at a ratio of 10:1 (v/w) for leaves, flowers and stage 1-2 fruit or 6:1 for more mature fruit. Samples were vortexed and incubated at 65°C for 30 min with vigorous mixing every 5 min. Following incubation, samples were centrifuged (4°C, 15 min, 3000 × *g*) and filtered through miracloth (Merck Chemicals Ltd., Nottingham, UK) prior to a second centrifugation at 13000 × *g* for 30 min (4°C). The sample supernatant was combined with an equal volume of chloroform:isoamyl alcohol (24:1) and mixed by inversion. Phase separation was achieved by centrifugation (13000 × *g*, 5 min, 4°C) and the upper aqueous layer extracted a second time against chloroform:isoamyl alcohol. Carbohydrates and nucleic acids were precipitated by the addition of 0.1 volumes 3 M sodium acetate and 0.6 volumes propan-2-ol followed by incubation at -80°C for 30 min. Samples were centrifuged (30 min, 4°C, 3500 × *g*) and pellets resuspended in 10 mM tris-HCl pH 7.5 containing 1 mM EDTA. Nucleic acids were selectively precipitated by the addition of 10 M LiCl to give a final concentration of 2 M and incubated overnight at 4°C. The RNA pellet was then recovered by centrifugation (16,500 × *g*, 30 min, 4°C), washed once in ice-cold 70% ethanol, air dried and resuspended in sterile diethyl pyrocarbonate treated water. Contaminating DNA was removed by DNAase treatment (Qiagen, West Sussex, UK) and further purified using RNeasy spin columns (Qiagen) according to the manufacturer's protocol. Sample concentration and purity was checked using a NanoDrop ND-1000 spectrophotomer (NanoDrop, DE, USA).

cDNA synthesis and PCR reactions

cDNA was synthesised using Ready-to-go You Prime First Strand Beads (GE Healthcare, Buckinghamshire, UK) from 10 µg total RNA using pd(N)6 random hexamers as primer.

Quantitative RT-PCR was performed with the primers described in **Table 1** at a final concentration of 3 nM using the Qiagen Quantitect SYBR Green PCR kit. cDNA templates (1 µl of a 10-fold dilution) were amplified in 25 µl using a Chromo4 Real-Time PCR Detector (Biorad, Hertfordshire, UK) with DNA amplification measured by fluorescence emission (521 nm) following excitation at 494 nm following each round of amplification. Thermal cycling conditions were 15 min denaturation at 95°C followed by 40 cycles (15 s at 95°C, 30 s at 58°C, 30 s at 72°C). Relative expression levels were calculated using 18S ribosomal RNA as control and the primers validated using the ΔΔCt method (Livak 1997). At the end of the PCR, melting curves were run to ensure product consistency.

Products for gene sequencing were amplified in 50 µl PCR reactions containing 50-100 ng of template DNA, 20 mM Tris-HCl pH 8.4, 2 mM $MgCl_2$, 50 mM KCl, 500 nM of each primer, 200

Table 1 Primers used for PCR reactions

Primer[a]	Function[b]	Sequence	Target
GDPMP F1	S (54°C)	CGATTCGTAGAAAAACCAAA	GDP-mannose pyrophosphorylase
GDPMP R1	S	AACTCCCGACTCAACCACAC	
GDPMP F2	S (54°C)	TCTGATCGGACCTGATGTTG	
GDPMP R2	S	CAAATGATCCAAACTTGCTGAA	
GDPME F1	S (55°C)	TTGATTTCTCAGCATCTCAAGG	GDP-mannose 3,5-epimerase
GDPME R1	S	GCACTCAATTCCGAAATCCT	
GDPME F2	S (57°C)	AACGGAGGAGTTGTGCAAG	
GDPME R2	S	TTGGGCTTTCAAAGAACCTC	
GDPMP F	Q	TTGCATCTCAGGCAGTATCA	GDP-mannose pyrophosphorylase
GDPMP R	Q	CACACCACCATTGCTGTAGA	
GDPME F	Q	AAGTTTGAGATGTGGGGTGA	GDP-mannose 3,5-epimerase
GDPME R	Q	TCATGCTAACCATTTCGTCA	
GDPGP F	Q	TGTTACTGCCTGTGAAACCA	GDP-L-galactose pyrophosphatase
GDPGP R	Q	GCTGGAGGACCTTATCAACA	
GalP F	Q	CATCTCAAGGAGCAGTACCC	L-galactose-1-P phosphatase
GalP R	Q	CCCATGGACGAAGTTAGTTG	
18S F	Q	TGGAAGGGACGCATTTATTA	Ribosomal 18S RNA
18S R	Q	GGGCAGAAATTTGAATGATG	

[a] F = forward primer, R = reverse primer
[b] S = primer used for sequencing reactions (numbers in brackets represent annealing temperature of PCR reactions), Q = primer used quantify relative gene expression based on SYBR Green fluorescence

µM of each dNTP and 1 unit of *Taq* DNA polymerase (Promega, Hampshire, UK). Thermal cycling conditions were 3 min denaturation at 95°C followed by 35 cycles of 30 s at 95°C, 1 min at the appropriate annealing temperature (**Table 1**) and 1 min at 72°C. Following the final cycle samples were maintained at 72°C for 5 min.

Sequencing reactions

Following PCR, sample products were purified using the Qiagen QiaQuick gel extraction kit according to the manufacturer's protocol. Samples were then sequenced in both the forward and reverse directions using a cycle sequencing protocol and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed on a 377 automated DNA sequencer (Applied Biosystems).

RESULTS

Growing season and location impact fruit AsA content

To determine the impact of growing season on fruit ascorbic acid content in blackcurrant, historical records available from cultivar selections at SCRI were interrogated. The available data covered the years 1972-2007 and for each cultivar a minimum of 20 years' fruit AsA data was available. All cultivars showed a similar trend in fruit AsA concentration, being high in the mid-1970's before falling to minimum values in the early 1990's and then increasing again up to the present day (**Fig. 1**). Differences between minimum and maximum ascorbate values were over 4-fold in cvs. 'Ben Alder' and 'Baldwin', approximately 3.5-fold in cv. 'Ben Lomond' and 2.5-fold in cv. 'Tirran'. Cultivar hierarchy with respect to fruit AsA content was generally maintained with cvs. 'Ben Lomond' and 'Baldwin' having a higher fruit ascorbate content than cvs. 'Ben Tirran' and 'Ben Alder'. Although the methodology for AsA quantification changed in 1994, there was no abrupt change in the AsA values recorded, therefore we conclude that the two methods used were comparable.

In addition to growing season, growing location impacted on fruit AsA content in cv. 'Ben Hope' where significant differences were observed between fruit grown in the Scottish central belt, south west and south east England (**Fig. 2**).

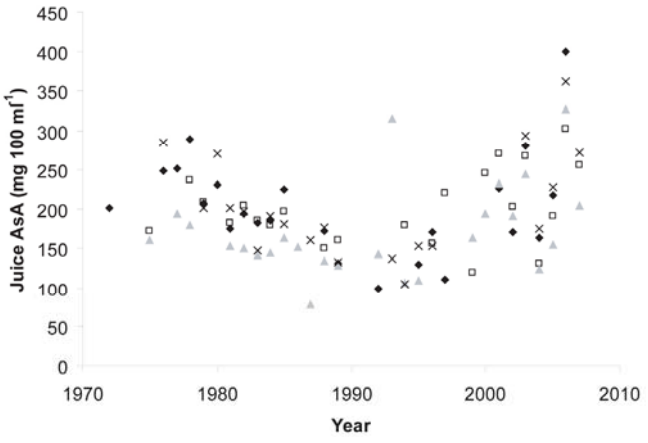


Fig. 1 Impact of growing season on ascorbic acid content of blackcurrant juice from four cultivars. Fruits were harvested from cultivars Baldwin (♦), Ben Tirran (□), Ben Alder (▲) and Ben Lomond (×) at commercial ripeness and juice extracted as described. Juice AsA concentration was determined by titration in samples harvested between 1972 and 1993 and by HPLC from 1994 onwards.

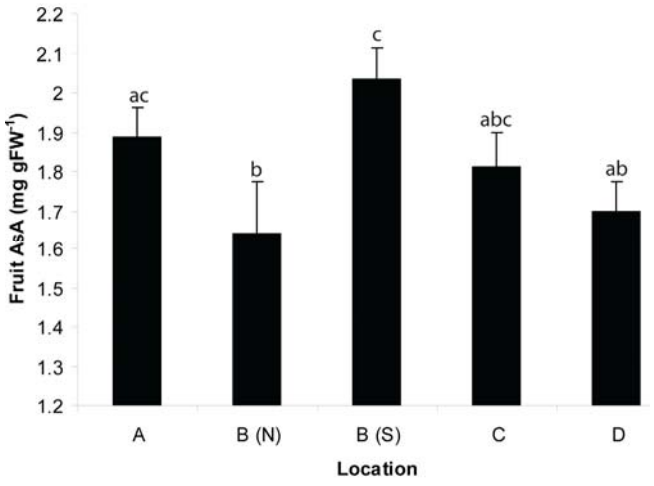


Fig. 2 Impact of growing location on fruit ascorbic acid content of blackcurrants grown in the same season. Fruit were grown in (**A**) Somerset (51°00′N, 3°11′W); (**B**) Angus (56°30′N, 3°05′W); (**C**) Kent (51°05′N, 0°30′E) and (**D**) Norfolk (52°39′N, 0°50′E). In Angus, fruit were collected from plants growing on north (N) or south (S) facing slopes. AsA was extracted from whole fruits in metaphosphoric acid and quantified by HPLC as described. Data are represented as mean values ± standard error (n = 5). Where bars are annotated with the same letter, they were not significantly different to one another as determined by the students' *t*-test ($p < 0.05$).

Aspect also had a significant impact on fruit AsA content with fruit grown on a south facing slope having nearly 25% more AsA than fruit grown on a north facing slope at the same location.

Correlation between environmental variables and fruit AsA content

In order to further understand the relationship between environmental conditions and fruit AsA content, meteorological records collected at SCRI were consulted in order to correlate meteorological conditions with fruit AsA. Pearson correlation coefficients were determined between fruit AsA content and total precipitation (mm), average air temperature (°C) and total solar radiation (mJ m^{-2}) over monthly, quarterly and annual periods. Overall, the strongest correlations were versus quarterly figures and therefore only the quarterly correlation data is presented here.

Correlations between total quarterly precipitation and fruit AsA content were low, generally having Pearson correlation coefficients below 0.3 (**Fig. 3A**). In addition, there was variation in the response of different cultivars to rainfall at different times of the year with cvs. 'Baldwin' and 'Ben Alder' showing weak positive correlations between fruit AsA content and total rainfall in the quarter August to October while cvs. 'Ben Tirran' and 'Ben Lomond' showed weak negative correlations for the same period. Taken together the data suggest that precipitation has little influence on fruit AsA content under the prevailing meteorological conditions of the northern United Kingdom over the past 35 years.

Average temperature had a slightly stronger influence on fruit AsA concentration, particularly in cv. 'Ben Lomond' where Pearson correlation coefficients were close to or in the case of the quarter April to June in excess of 0.5 (**Fig. 3B**). Positive correlations were observed for most cultivars for most dates however, cv. 'Baldwin' showed a weak negative correlation against average temperature during the late winter and early spring periods (January – March, February – April, Mar – May).

Some of the strongest correlations observed were between total solar radiation and fruit AsA content and this was particularly noticeable when three month periods during flowering and fruit development were taken (February – April, March – May, April – June, May – July, June – August, July – September) (**Fig. 3C**). Throughout these months, the relationship between total solar radiation and fruit ascorbate content was positive. Correlations were strongest in cvs. 'Ben Tirran' and 'Ben Lomond' with cv. 'Baldwin' also showing strong positive correlations particularly later in the season (May – July, June – August, July – September).

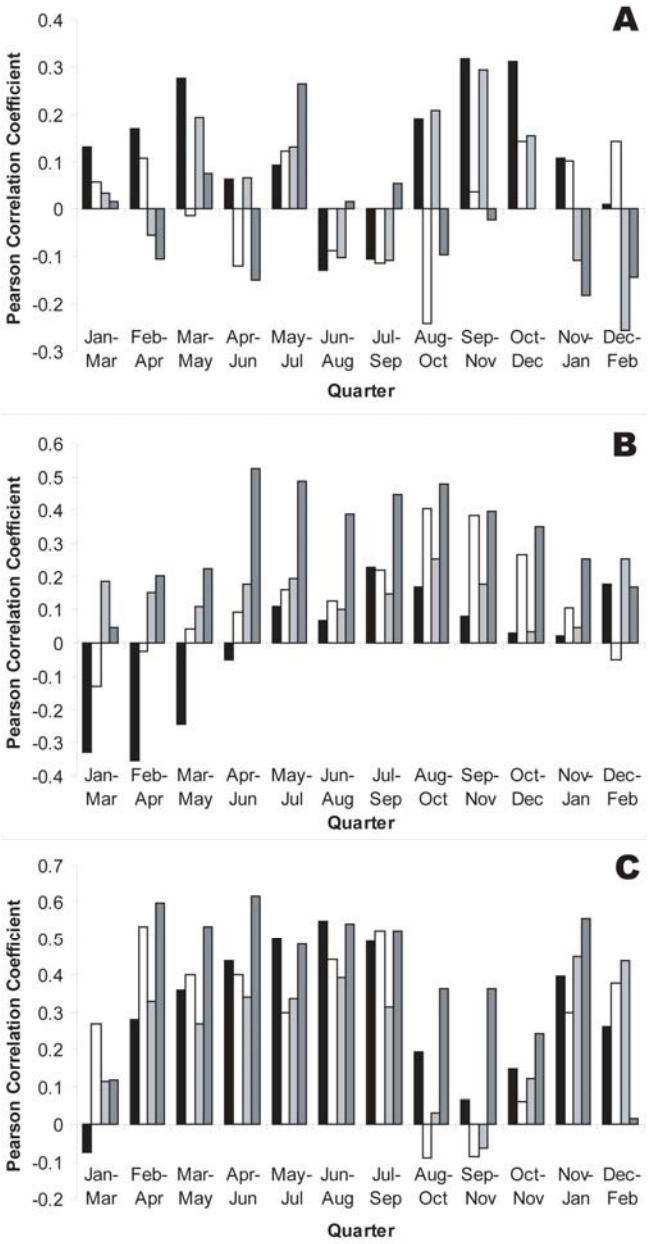


Fig. 3 Pearson correlation coefficients between juice AsA content and meteorological variables for four blackcurrant cultivars. Juice ascorbic acid content was correlated against (**A**) total precipitation (mm); (**B**) average daily temperature (°C) or (**C**) total solar radiation (mJ m^{-2}) in the four cultivars Baldwin (■), Ben Tirran (□), Ben Alder (■) and Ben Lomond (■). Correlations were performed for each three month period in the year up to fruit harvest at one month intervals so that 12 correlations were performed for each cultivar.

AsA accumulation does not correlate with the expression of key genes of the L-galactose pathway

Previously, we demonstrated that AsA accumulation occurs in blackcurrant fruit primarily by *in situ* biosynthesis via the L-galactose pathway and that biosynthetic capacity is high in flowers and expanding fruit (stages 1-2) but rapidly declines as fruit mature further (stages 3-6) (Hancock *et al.* 2007, **Fig. 4C inset**). In order to determine the relationship between AsA biosynthesis and gene expression in blackcurrant fruit, key genes encoding biosynthetic enzymes were examined throughout fruit development. The genes chosen for expression studies were those considered to either control rate limiting steps or those that are unique to the L-galactose pathway for AsA biosynthesis. Gene expression was monitored in fruit of three different genotypes; 8982-6 containing 270 mg AsA 100g FW^{-1}, cv. 'Baldwin' containing 220 mg AsA 100 g FW^{-1} and cv. 'Hedda' containing 60 mg AsA 100 g FW^{-1} at harvest respectively (**Fig. 8**).

GDP-D-mannose pyrophosphorylase (E.C. 2.7.7.22, GDPMP) is required for the synthesis of GDP-D-mannose from GTP and D-mannose-1-phosphate. Mutations in the *vtc1* gene of *Arabidopsis thaliana* resulted in a 75% reduction in plant AsA content associated with a 35% reduction in GDPMP activity (Conklin *et al.* 1999). Furthermore potato plants harbouring an antisense GDPMP construct had up to 56% reduction in leaf AsA content associated with a similar reduction in enzyme activity (Keller *et al.* 1999). These data suggest that GDPMP exerts significant control over ascorbate biosynthesis. GDPMP gene expression was low in blackcurrant flowers for all cultivars examined. Expression levels increased during early fruit development to reach maximum levels of expression in fruit stages 2–3 before falling to lower levels as fruit matured (**Fig. 4A**). Of the four genes examined, GDPMP was the most differentially expressed with up to 13-fold variation in expression during development in cv. 'Baldwin', 12-fold in cv. 'Hedda' and 6-fold in genotype 8982-6.

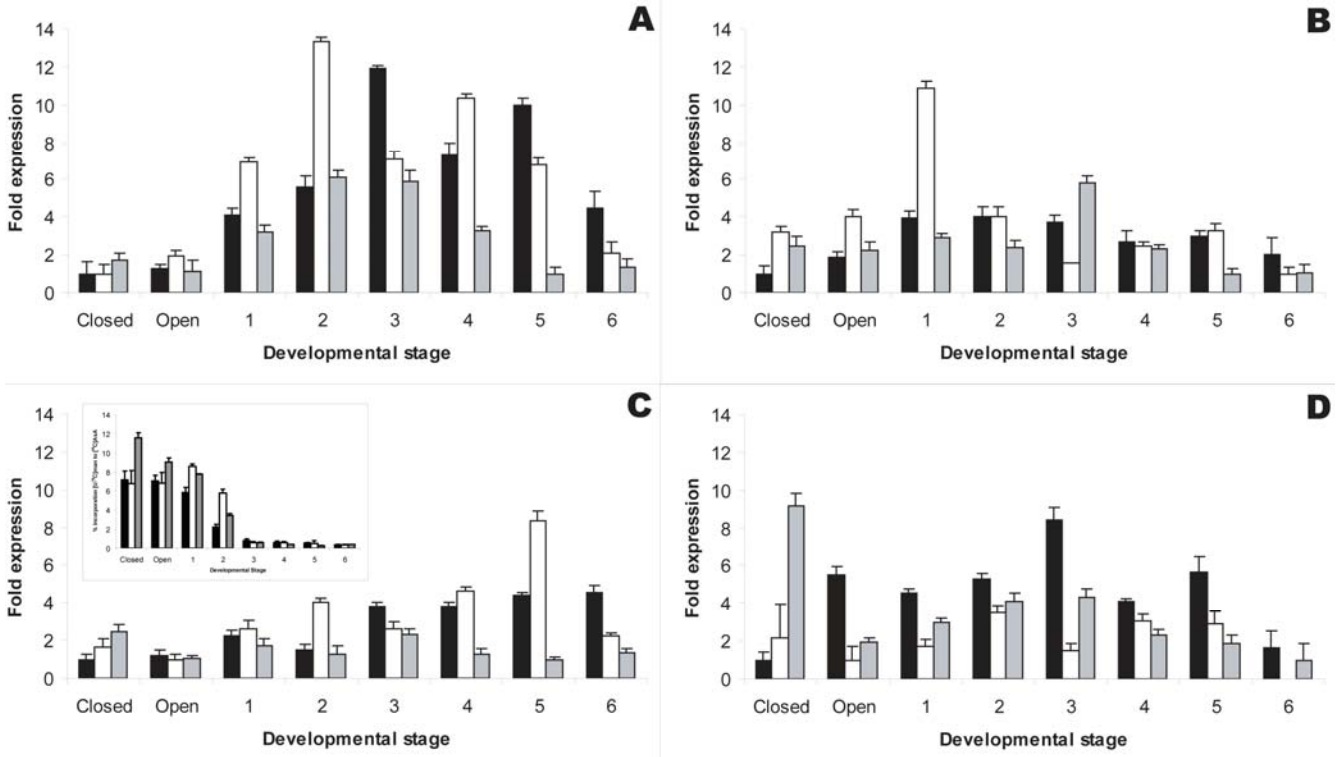


Fig. 4 Expression analysis of four key genes of the L-galactose pathway of AsA biosynthesis in developing fruit of three blackcurrant genotypes. Expression of genes encoding (**A**) GDPMP; (**B**) GDPME; (**C**) GDPGP and (**D**) GalPase was monitored in the cultivars Hedda (■) and Baldwin (□) and genotype 8982-6 (■) by RT-PCR relative to the expression of the 18S mRNA subunit. All data are expressed as fold change in gene expression. Inset in (**C**) shows fruit biosynthetic capacity as estimated from the incorporation of d-[U-^{14}C]mannose to [^{14}C]AsA (Hancock *et al.* 2007).

GDP-D-mannose 3,5-epimerase (E.C. 5.1.3.18, GDPME) catalyses the conversion of GDP-D-mannose to GDP-L-galactose and GDP-L-gulose *in vitro* (Wolucka and van Montagu 2003). *In vivo*, its primary function is the synthesis of GDP-L-galactose which is further metabolised to AsA (Linster and Clarke 2008) with smaller quantities providing L-galactosyl residues in cell walls (Seifert 2004). Analysis of AsA QTLs in three tomato populations showed that a gene encoding GDPME co-located with a QTL identified in all three populations (Stevens *et al*. 2007) suggesting that alleles of this enzyme may affect fruit ascorbate content and indicating its potential role in the control of AsA accumulation. Expression patterns for GDPME were similar to those observed for GDPMP in that they were low in flowers, rising in the mid-ripe fruit stage before falling away as fruit ripened further (**Fig. 4B**).

GDP-L-galactose phosphorylase (GDPGP) catalyses the phosphorylation of GDP-L-galactose to form GDP and L-galactose-1-phosphate (Linster *et al*. 2008) and can be considered the first committed step in ascorbate biosynthesis. Two genes encode the activity in *A. thaliana* and they have been shown to be under the control of both light and the circadian clock (Dowdle *et al.* 2007). Furthermore, transient overexpression of a gene encoding GDPGP activity from kiwifruit in tobacco leaves resulted in a three fold increase in foliar ascorbate levels (Laing *et al*. 2007). All of these data suggest that the enzyme may play a key role in controlling AsA biosynthesis in plants. In cvs. 'Hedda' and 'Baldwin' gene expression was low in flowers rising to a maximum at developmental stage 6 in cv. 'Hedda' and stage 5 in 'Baldwin'. In genotype 8982/6, expression levels were highest in closed flowers although there was little differential expression of the gene throughout development (**Fig. 4C**).

L-Galactose-1-phosphate phosphatase (GalPase) catalyses the hydrolysis of L-galactose-1-phosphate to free L-galactose and inorganic phosphate. As several studies have shown that supply of L-galactose to plant tissues results in a rapid accumulation of AsA (Hancock and Viola 2005) this step in the biosynthetic pathway could be considered the last opportunity for control over AsA accumulation. The importance of the enzyme in controlling tissue AsA content is highlighted from analyses of *A. thaliana vtc* mutants. The *vtc4-1* mutation contained only 40% of wild-type AsA levels and was mapped to a gene encoding a putative GalPase resulting in a two fold decrease in activity in partially purified enzyme extracts (Conklin *et al.* 2006). In cv. 'Hedda' gene expression was low in closed flowers but increased 5.5-fold in open flowers and maintained high until fruit developmental stage 5. In cv. 'Baldwin', differential expression was limited with gene expression lowest in open flowers. In genotype 8982/6 expression was highest in closed flowers with a second peak of expression at mid-fruit ripening stages (2–3) (**Fig. 4D**).

Cloning and characterisation of *Ribes nigrum* GDPMP and GDPME

Given the lack of any correlation between gene expression of 4 key genes of the L-galactose biosynthetic pathway for AsA and fruit AsA accumulation, further analysis was undertaken to determine whether differences in AsA accumulation could be accounted for by differences in the gene coding sequence between cultivars. Initially sequences from a blackcurrant cDNA library constructed from green/red Ben Alder fruit (Woodhead *et al*. 1998) were screened and sequences were identified corresponding to GDPMP and GDPME.

The cDNA insert ERibFSQ01_N03 (965 bp; Accession number: FJ546463) contained a partial cDNA sequence with homology to GDPMP. The cDNA was predicted to encode a polypeptide of 261 amino acids which was truncated by approximately 100 amino acids at the N-terminus when compared against annotated GDPMP's using the MUSCLE multiple alignment programme (Edgar 2004) (**Fig. 5**). The PFAM protein family database (Finn *et al.* 2008) identified the truncated protein as a member of the nucleotidyl transferase family catalysing the transfer of nucleotides from nucleotide triphosphates to sugar phosphates. The truncated blackcurrant protein was most closely related to those from

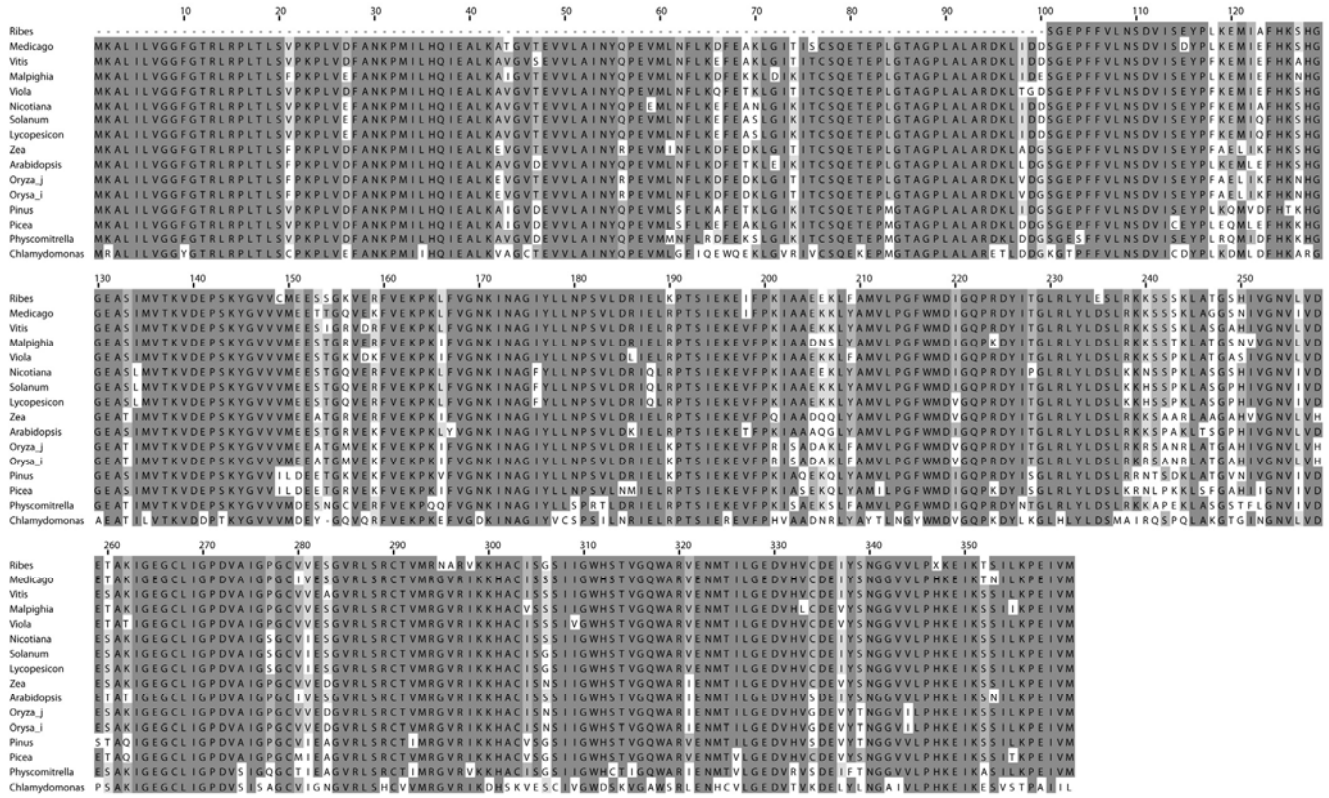


Fig. 5 Multiple alignment of amino acid sequences of GDPMP from plants, moss and algae. Peptide sequences from *Ribes nigrum*, *Medicago sativa* (Q6DWO8), *Vitis vinifera* (A7PIN5), *Malpighia glabra* (A0EJL9), *Viola baoshanenis* (Q09PG3), *Nicotiana tabacum* (Q94IA7), *Solanum tuberosum* (Q9ZTW5), *Lycopersicon esculentum* (Q6J1L7), *Zea mays* (B4FEP8), *Arabidopsis thaliana* (O22287), *Oryza sativa* subsp. *japonica* (Q6Z9A3), *Oryza sativa* subsp. *indica* (A2YSQ4), *Pinus taeda* (A6N835), *Picea sitchensis* (A9NUV9), *Physcomitrella patens* (A9TU90) and *Chlamydomonas reinhardtii* (A8J3F9) were aligned using the MUSCLE programme. The strength of residue identity amongst sequences is indicated by the intensity of grey highlighting.

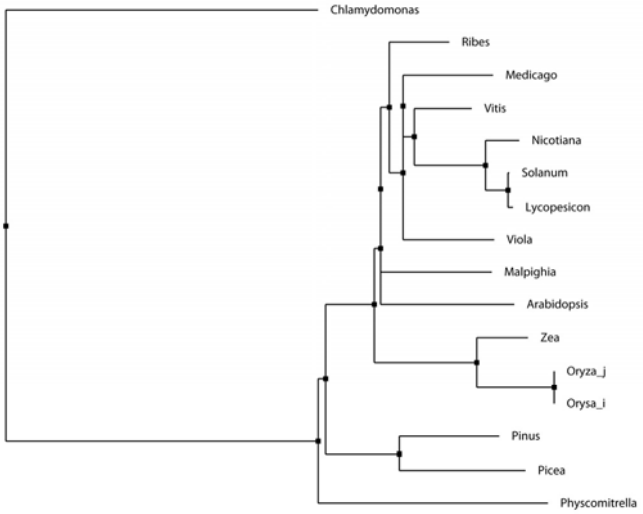


Fig. 6 Neighbour joining phylogenetic tree for GDPMP. The phylogenetic analysis was performed within Jalview (Clamp *et al.* 2004) using % identity. The sequences used were those described in the legend for **Fig. 5**.

other dicotyledonous plants including medicago (91% identity, E=7e-127), grapevine (91% identity, E=1e-126), potato (89% identity, E=5e-126) and tomato (89% identical, E=6e-127). The monocotyledonous plants formed a separate clade while the protein from trees (Sitka spruce, Loblolly pine), moss and algae were more distantly related (**Fig. 6**).

The cDNA insert for GDPME (ERibF_SQ01_K04; Accession number: FJ546462) was 1534 bp in length with a small 5′-untranslated region (UTR) from position 1-83 bp, a start codon at 84 bp and a stop codon at 1211 bp, with a 3′UTR of 322 bp. A BlastP search indicated the blackcurrant sequence showed greatest homology to GDPME peptide sequences from grape (94% identity, E=7.4e-196) and acerola (*Malpighia glabra*; 94% identity, E=2.2e-196). The sequence encoded a polypeptide of 376 amino acids with a calculated molecular mass of 42568 Da and a theoretical pI of 5.88. The SOSUI programme (Hirokawa *et al.* 1998) predicted the protein to be soluble while the SignalP programme (Bendtsen *et al.* 2004) failed to predict signal peptides and iPSORT (Bannai *et al.* 2002) predicted a cytoplasmic location. The PFAM protein family database (Finn *et al.* 2008) identified the predicted protein as a member of the NAD dependent epimerase/dehydratase family with the predicted active site residues S143, Y173, K177.

The predicted protein showed strong homologies to predicted proteins from other dicotyledonous and monocotyledonous plants, a moss and a unicellular green alga. The full sequence alignment between the *R. nigrum* and *A. thaliana* proteins are shown in **Fig. 7A**. All of the sequences examined had a conserved GxxGxxG NAD binding motif (Bellamacima 1996) although with the exception of the monocotyledonous plants and *Mesembryanthemum crystallinum* the final glycine of the motif was replaced with an alanine as has been previously observed (Watanabe *et al.* 2006). The sequences also exhibited the short chain dehydrogenase/reductase catalytic motif YxxxK (Y173, K177 of the blackcurrant protein) with an upstream catalytic serine corresponding to the S143 of the blackcurrant sequence (Jornvall *et al.* 1995) (**Fig. 7B**). The blackcurrant protein was most strongly related to those obtained from other shrubs and trees (Sitka spruce, *Picea sitchensis*; grapevine, *Vitis vinifera* and acerola cherry, *Malpighia glabra*). Other clades were formed among the grasses, the solanaceae and other dicotyledonous plants. The proteins from moss (*Physcomitrella patens*) and green algae (*Chlamydomonas reinhradtii*) were more distantly related (**Fig. 7C**).

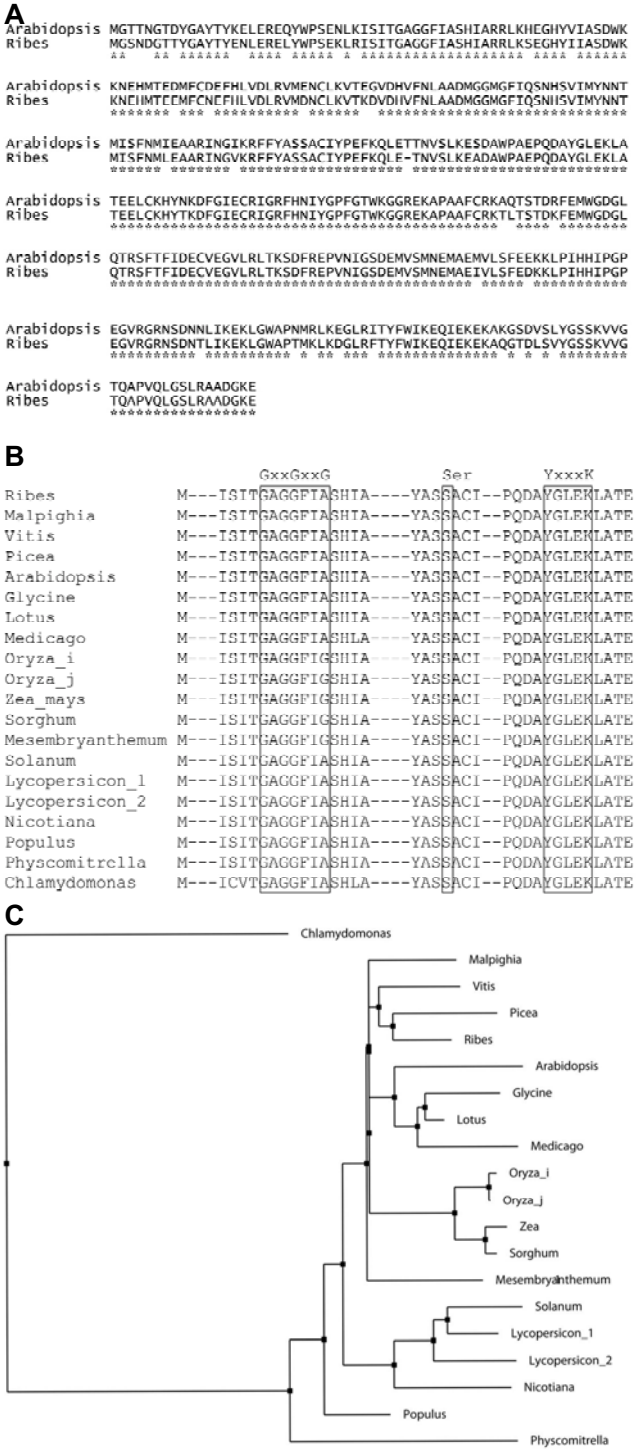


Fig. 7 Amino acid sequence and phylogenetic analysis of GDPME. (**A**) Pairwise amino acid sequence alignment of *Arabidopsis thaliana* (Q9V3R3) and *Ribes nigrum* GDPME proteins. Gaps (-) were introduced to achieve maximum similarity and identical residues are marked by an asterisk. (**B**) Motif sequence alignments of GDPME proteins from higher plants, a moss and an alga. The NAD-binding GxxGxxG motif and catalytic serine (S), tyrosine (Y) and lysine (K) residues are highlighted. Predicted protein sequences used in the alignment were from *Ribes nigrum*, *Malpighia glabra* (A0EJL8), *Vitis vinifera* (A5JPK5), *Picea sitchensis* (A9NUD9), *Arabidopsis thaliana* (Q9V3R3), *Glycine max* (CAD70060), *Lotus japonicus* (CAD70066), *Medicago trunculata* (CAD70062), *Oryza sativa* subsp. *indica* (A2Z7B3), *Oryza sativa* subsp. *japonica* (A3C4S4), *Zea mays* (B4FBC2), *Sorghum bicolour* (CAD70063), *Mesembryanthemum crystallinum* (CAD70059), *Solanum tuberosum* (Q2XPW6), *Lycopersicon esculentum* (CAD70057, CAD70067), *Nicotiana tabacum* (CQ809292), *Populus trichocarpa* (A9PGJ0), *Physcomitrella patens* (A9TIB8) and *Chlamydomonas rein-hardtii* (A8HPS2) (**C**) A neighbour joining tree derived from GDPME amino acid sequences from a number of plants. The phylogenetic analysis was performed within Jalview (Clamp *et al*. 2004) using % identity. The sequences used in the analysis were as described for **Fig. 7B**.

Analysis of GDPMP and GDPME coding regions in a range of blackcurrant genotypes

Previous work has demonstrated that blackcurrant genotypes have a broad range of AsA contents (Viola *et al.* 2000; Walker *et al.* 2006; Hancock *et al.* 2007), however data presented here suggests that such differences cannot be correlated with the expression of key genes encoding AsA biosynthetic enzymes (**Fig. 4**). Therefore, experiments were undertaken to determine whether genetic polymorphism within the coding regions of GDPMP or GDPME could be correlated with fruit AsA content.

Approximately 40 blackcurrant genotypes from the SCRI breeding programme were phenotyped for ripe fruit ascorbate content (**Fig. 8**). Plant material represented a wide range of phenotypes with juice ascorbate ranging from 50 mg 100 ml juice^{-1} in genotype 9448-4 to 270 mg 100 ml juice^{-1} in genotype 8992-11 with a mean value of 149 mg 100 ml^{-1} and a standard deviation about the mean of 61 mg 100 ml^{-1}.

RNA was extracted from leaves and reverse transcribed to create a cDNA template for PCR sequencing reactions. Two overlapping primer pairs were designed to allow sequencing of GDPMP cDNA (**Table 1**). Readable sequence was returned for the DNA encoding amino acids 174–361 as shown in **Fig. 5** with the exception of residues 283–285. The nucleotide sequence was completely conserved to that of Ben Alder (**Fig. 5**) in all genotypes with the exception of genotype 8999-9 in which there was a single nucleotide A → T change at position 739 corresponding to A246 (**Fig. 9**), outside of the region identified as belonging to the nucleotidyl transferase family. Genotype 8999-9 displayed a juice ascorbate content of 187 mg 100 ml^{-1}, slightly above the average value of all genotypes tested and the base change was not predicted to result in any change in the protein amino acid sequence.

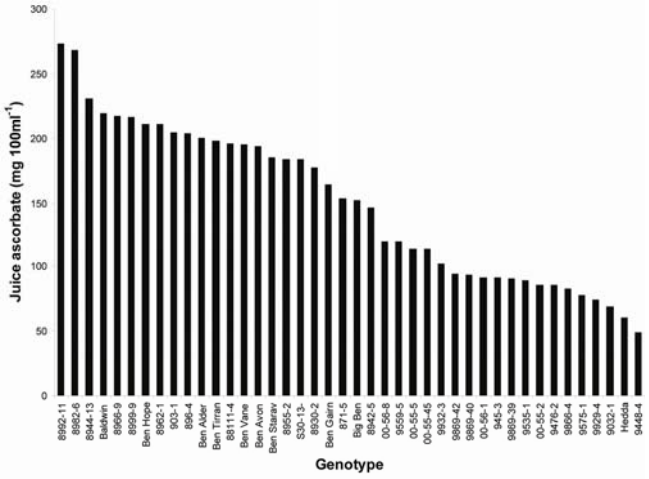
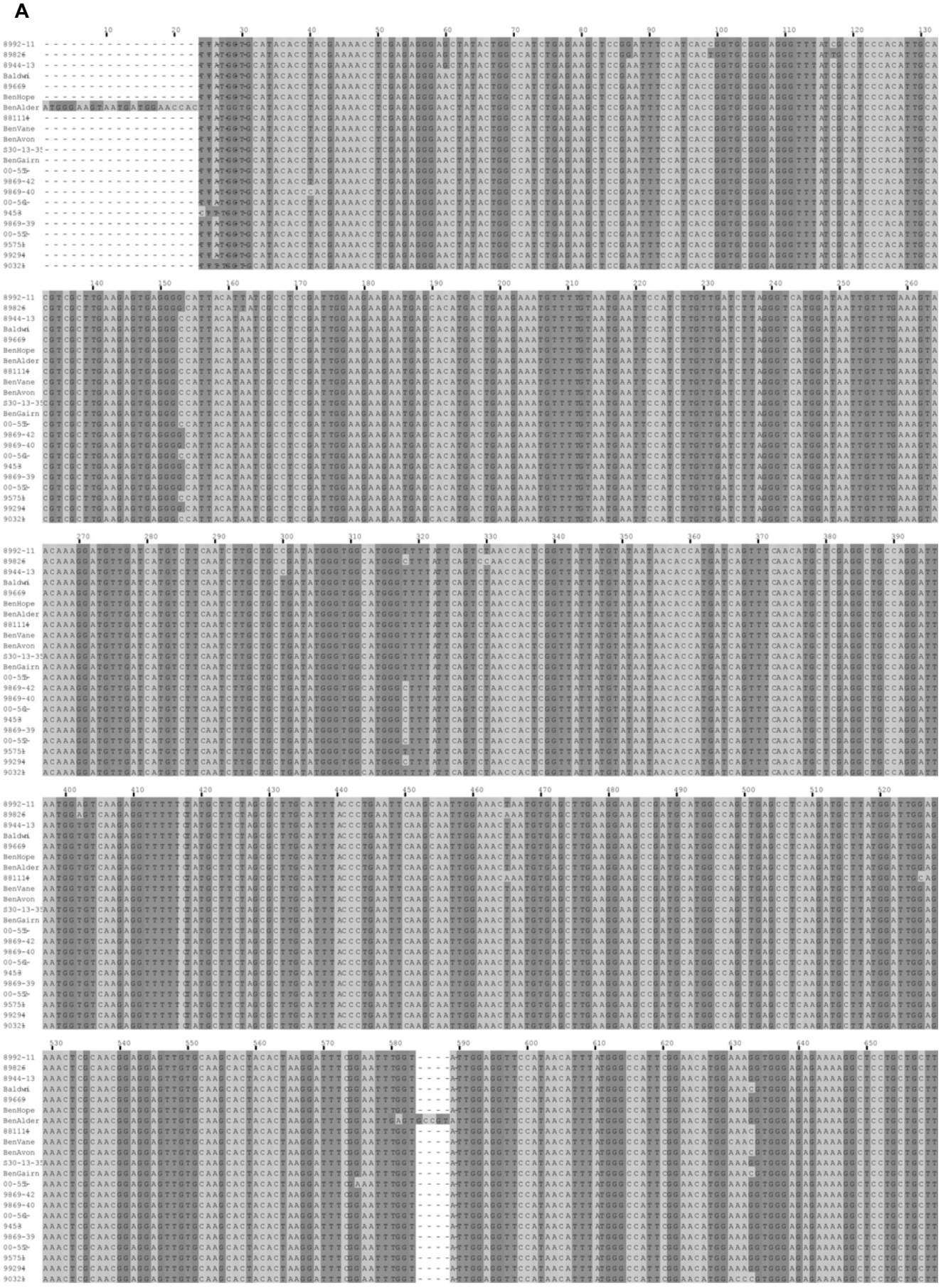


Fig. 8 Ascorbic acid content of blackcurrant genotypes. Juice ascorbic acid was measured at commercial ripeness in triplicate by HPLC from fruit harvested in 2005.

Fig. 9 Polymorphism within the GDPMP coding region of blackcurrant genotypes. Nucleotide sequence (residues 725–751) is shown with the corresponding predicted amino acid (residues 242–250) for a section of the GDPMP coding region in cv. Ben Alder and genotype 8999-9. The single A → T nucleotide substitution in 8999-9 is highlighted.

Similarly, overlapping primer pairs were designed for the GDPME sequence. In this case, readable sequence was returned for nucleotides 24–1127 with the exception of nucleotides 584–588 inclusive (**Fig. 10A**) allowing translation of amino acids 9–376 with the exception of amino acids 194–196 inclusive (**Fig. 10B**). More genetic variation was observed within the coding sequence of GDPME with sequences from all individual genotypes with the exception of 8966-9, 'Ben Hope', S30-13-35 and 9575-1 differing from the consensus sequence constructed by comparison of



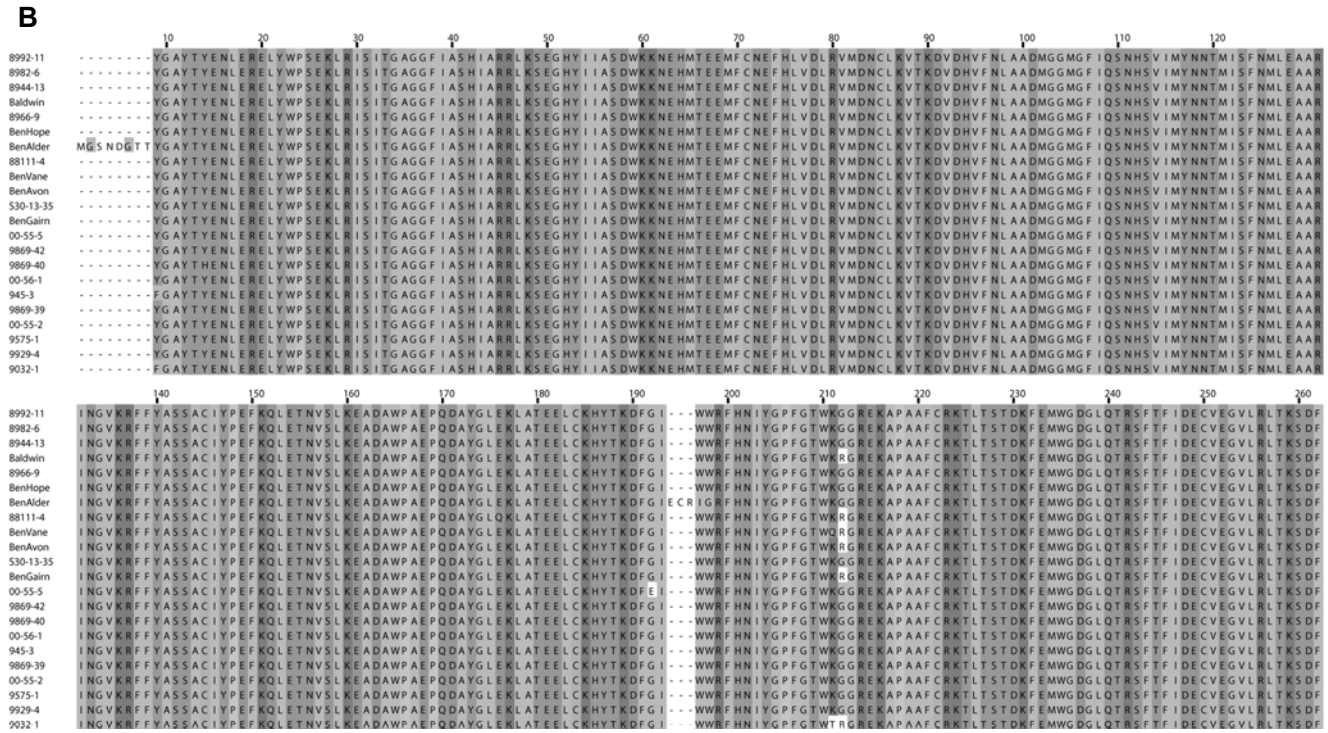


Fig. 10 Polymorphism within the GDPME coding region of blackcurrant genotypes. (**A**) Multiple nucleotide sequence alignment for GDPME cDNA of several blackcurrant genotypes. As no diversion from the consensus sequence was observed beyond nucleotide 633 only the first 670 bases are shown. Blackcurrant genotypes are listed to left of each line and are represented from high to low fruit ascorbate content. (**B**) Multiple amino acid sequence alignment for GDPME proteins of several blackcurrant genotypes. As no diversion from the consensus sequence was observed beyond amino acid 212 only the first 262 amino acids are shown. Blackcurrant genotypes are listed to left of each line and are represented from high to low fruit ascorbate content.

the sequence from 22 genotypes in at least one base. Variation was primarily observed within the 5′ region of the coding sequence and there was no deviation from the consensus sequence after nucleotide 633 corresponding to G/R 212. In only ten of the 22 genotypes did nucleotide substitutions result in changes in the protein amino acid sequence and no variations in the catalytic amino acids S143, Y 173 of K177 were observed. Similarly, the NAD binding motif was conserved in all genotypes with the final glycine substituted for alanine as observed in other dicotyledenous plants (**Fig. 7B**).

The three genotypes determined to have the highest concentration of AsA in juice shared a number of sequence variations; an A → G substitution at nucleotide 60, an A → C substitution at nucleotide 120 and a C → T substitution at nucleotide 300. In addition, the top two genotypes had an A → G substitution at nucleotide 87, an A → T substitution at nucleotide 162 and a T → A substitution at nucleotide 402. These were all synonymous substitutions (i.e. these changes did not alter the predicted amino acid sequence of the translated genes).

DISCUSSION

Genetics and environment contribute to control of fruit AsA content

Analysis of fruit AsA content of four cultivars grown for 35 years at the same location revealed large annual differences within individual cultivars (**Fig. 1**). All cultivars showed the same trends in fruit AsA concentration over time and cultivar hierarchy was generally maintained. These data suggest that i) prevailing environmental conditions impact fruit AsA content, ii) the four cultivars analysed all respond to environmental conditions in a similar fashion and iii) genetic background impacts fruit AsA content under a range of environmental conditions. The finding that environment impacts on fruit AsA content was further supported by data revealing significant differences in AsA content of fruit from cv. 'Ben Hope' grown at different locations in the same year (**Fig. 2**).

Analysis of meteorological records suggested environmental interactions were complex with relatively low correlation coefficients between single meteorological variables and fruit ascorbate content. Nevertheless, solar radiation during the months of berry development consistently returned correlation coefficients with fruit AsA content in excess of 0.3 across all cultivars tested (**Fig. 3**). Similarly, fruit from bushes grown on a south facing slope contained 20% more AsA than fruit from bushes grown on a north facing slope at the same location. Although not directly recorded, it would be expected that fruit on the south facing slope would receive more solar radiation than those grown on the north facing slope. It is of course possible that other environmental variables may also be altered, however automated temperature measurements from data loggers placed at the base of each plant revealed that this variable was unchanged between the two conditions (data not shown). Previous work has shown wide annual variation in AsA content dependent on growing season in a variety of fruit including apples (Łata *et al*. 2005), tomatoes (Toor *et al.* 2006) and strawberries (Hakala *et al.* 2003). In fact it appears that fruit AsA content is phenotypically flexible and highly responsive to environmental conditions. Previous work has shown that temperature is negatively correlated with fruit AsA content in blackcurrants (Redlen 1993), kiwifruit (Richardson *et al*. 2004; Snelgar *et al.* 2005) and tomato (Riga *et al.* 2008) under controlled conditions. However in the current study correlations between mean annual, quarterly or monthly temperatures were low and mostly positive. This may have been as a result of the complex interaction of other environmental factors under uncontrolled conditions. Alternatively, considering the northerly location of the blackcurrant plots used in the analysis, there may be a threshold temperature below which temperature has little impact.

In our analysis, there was very little influence of total precipitation on fruit AsA content under ambient conditions.

These results are not particularly surprising given that in most years natural precipitation in Scotland is adequate for blackcurrant development. Furthermore, studies using controlled deficit irrigation in fruit have shown mixed effects with deficit irrigation failing to have any impact on the AsA content of greenhouse grown tomatoes (Mahajan and Singh 2006) or eight out of nine field grown watermelon cultivars (Leskovar *et al.* 2004). On the contrary, water stress was demonstrated to have a negative impact on the AsA content of glasshouse grown cherry tomatoes (De Pascale *et al.* 2007) and field grown peach (Buendía *et al.* 2008).

Solar radiation has been frequently positively associated with fruit AsA content; for example sun exposed exocarp of apple fruit has been shown to contain significantly higher levels of ascorbate than shaded exocarp from the same fruit suggesting that the light dependent control of AsA accumulation arises within the fruit itself (Davey *et al.* 2004; Li *et al.* 2008). Similar results were obtained from tomato where the south facing and most sun exposed fruit quadrant contained 16% more AsA than the north facing quadrant (Venter 1977). Several shading studies have also suggested a role for light intensity in controlling the AsA content of tomato fruit. Early work demonstrated that transfer of tomato plants to shade at any time to the mature green fruit stage resulted in a 40% reduction in fruit AsA at maturity (Hamner *et al.* 1945) and several authors have demonstrated a linear relationship between light intensity during fruit development and fruit AsA content at maturity (Venter 1976; El-Gizawy *et al.* 1992). Despite the strong correlation between light intensity and fruit ascorbate in these controlled experiments, enhancing the light received by field grown strawberry plants through the use of highly reflective mulches enhanced the AsA content of cv. 'Elsanta' but not of cv. 'Flamenco' fruit (Atkinson *et al.* 2006).

Fruit ascorbate content does not correlate with biosynthetic gene expression

Previously, we demonstrated a strong correlation between accumulation of AsA in blackcurrant fruit and biosynthetic capacity via the L-galactose pathway (Hancock *et al*. 2007). Therefore expression of key genes encoding biosynthetic enzymes was analysed by qRT-PCR in order to understand the underlying genetic basis of phenotypic differences in fruit AsA between genotypes. The cvs. 'Hedda', 'Baldwin' and genotype 8982-6 were chosen for investigation on the basis of their wide differences in fruit ascorbate (**Fig. 8**). Surprisingly, expression of genes encoding GDPMP, GDPME, GDPGP and GalPase showed remarkably little correlation with either fruit AsA biosynthetic capacity or phenotypic ranking. It was previously shown that blackcurrant fruit have a high capacity for AsA biosynthesis in the flower and early fruit stages (stages 1, 2) but that the biosynthetic capacity declines rapidly as the fruit mature further (Hancock *et al*. 2007) however, expression of the four genes examined tended to peak around the mid stages of maturity (stages 2–5). Furthermore, there was little correlation between levels of gene expression and fruit ascorbate content of the different genotypes.

GDPMP is a multifunctional enzyme important not only for ascorbate synthesis but also for the provision of mannosyl residues for cell wall polysaccharides and protein glycosyl groups (Reiter and Vanzin 2001). Previous studies have shown that GDPMP expression was not correlated with AsA accumulation in tobacco BY-2 suspension cultures but peaked during the logarithmic phase of cell expansion when cell AsA concentration was in decline (Tabata *et al.* 2002). These data suggest that in tobacco cell culture, GDPMP gene expression is more strongly correlated with its requirement for the provision of cell wall precursors. In tomato, low molecular weight glucomannan synthesis increased during ripening (Tong and Gross 1988) and the GDPMP expression pattern in blackcurrant may be related to similar processes in this fruit. On the contrary, in acerola a fruit with an exceptionally high ascorbate content of up to 35 mg gFW^{-1} (Johnson 2003) a decline in the AsA content of fruit throughout ripening was mirrored by a decline in GDPMP expression (Badejo *et al.* 2007). Similarly, in *Arabidopsis* leaves GDPMP expression was correlated with accumulation of AsA on transfer from low to high light (Yabuta *et al.* 2007). Therefore, in systems with a low mannose demand for incorporation into polysaccharides and protein glycosyl units it appears that GDPMP gene expression is closely correlated with AsA accumulation whereas in systems where there are greater demands for mannose from alternative pathways AsA related changes may be masked.

Like GDPMP, GDPME has dual functionality providing intermediates for AsA biosynthesis and for cell wall polysaccharides (Reiter and Vanzin 2001) and in the present study patterns of GDPME expression were similar to those observed for GDPMP however the levels of differential expression were considerably lower. This result could be interpreted as being consistent with the functional significance for differential expression again being related to changes in cell wall metabolism during fruit ripening. Previous studies have shown little relationship between GDPME expression and AsA accumulation in *Arabidopsis* leaves under differing light regimes (Yabuta *et al.* 2007) and while methyl jasmonate treatment of tobacco BY-2 cultures resulted in a transient increase of AsA concentration that was associated with an increase in GDPME expression, transcript levels of GDPME remained high even after AsA began to decline (Wolucka *et al.* 2005). These data combined with the data presented here suggest that GDPME expression has little impact on the control of AsA accumulation in plants.

The first committed step of the L-galactose pathway is the phosphorylation of GDP-L-galactose by GDPGP to produce L-galactose-1-phosphate (Dowdle *et al.* 2007; Laing *et al.* 2007; Linster *et al.* 2007). Previous work has shown that the gene encoding GDPGP in *Arabidopsis* (*vtc2*) is regulated by light and that high levels of expression are correlated with high AsA content (Dowdle *et al.* 2007; Müller-Moulé 2008). However, in our analysis of blackcurrant fruit, gene expression was unrelated to AsA biosynthetic capacity where expression of the gene encoding GDPGP tended to be higher later in fruit development as biosynthetic capacity declined. These data suggest post-transcriptional or post-translational control of enzyme activity in blackcurrant fruit however, the observation that a 50-fold increase in *in vitro* enzyme activity following transient expression of kiwifruit GDPDP in tobacco resulted in only a threefold rise in leaf AsA content (Bulley *et al*. 2009) suggests kinetic control. Why expression of the gene should rise as AsA biosynthesis falls is unknown although a recent observation that the *Arabidopsis* gene product is localized to both the cytoplasm and the nucleus suggests that the protein may be multifunctional (Müller-Moulé 2008).

GalPase showed a similar pattern of expression to GDPMP and GDPME with expression generally peaking at the mid-ripening stages co-incident with reduced AsA biosynthetic capacity. Little information is available regarding the expression of GalPase in other systems however there was a small increase in expression of the gene in *Arabidopsis* on transfer to continuous light that was associated with AsA accumulation (Yabuta *et al.* 2007) although extractable enzyme activity was the same in low and high light grown *Arabidopsis* despite a 1.5-fold increase in AsA content under high light conditions (Dowdle *et al*. 2007). Taken together these data suggest that GalPase expression exerts little control over tissue AsA content.

The finding that expression of none of the genes encoding key enzymes for AsA biosynthesis correlated with biosynthetic capacity in blackcurrant fruit suggests that biosynthetic control is post-transcriptional or post-translational. In a previous study we showed that soluble sugars begin to accumulate in fruit from ripening stage 2 up until fruit maturity (Hancock *et al.* 2007), and it therefore seems unlikely that control of AsA accumulation is controlled by substrate availability. Alternative mechanisms may include

control of translation rate, rate of protein turnover and kinetic control of enzyme activities. For example, GDPME is inhibited by GDP, GDP-sugars, AsA and reduced nictotinamide adenine dinucleotides but activated by oxidized nicotinamide adenine dinucleotides, furthermore enzyme activity may be modulated through protein-protein interactions (Wolucka and van Montagu 2003). Finally, it is interesting to note that in a study of transcriptomic and enzyme activity profiles of 23 enzymes involved in carbon and nitrogen metabolism in *Arabidopsis* following transition from 12 h light-dark cycles to continuous night resulted in a much greater reduction in transcript levels than in enzyme activity (Gibon *et al.* 2004).

Polymorphism in the GDPME coding region represents a potential marker for breeding high AsA cultivars

AsA content represents a key quality trait in blackcurrant fruit and as processors and consumers are becoming increasingly demanding there is a need to combine high fruit AsA content with other important quality (nutritional value, acidity, sensory characteristics) and agronomic (frost tolerance, pest/disease resistance) traits (Brennan and Gordon 2002). Combining all of the desired traits into a single genotype is a demanding and labour intensive process requiring the generation of many crosses which must then be propagated under glass prior to transfer to the field to be grown on until maturity to allow the determination of fruit phenotype in addition to growth habit, yields, performance in relation to different environments and pest/disease resistance. Processors demands are now such that even if a particular genotype performs in all other characteristics it will still be discarded if the fruit contains an unacceptably low AsA content. A predictive genetic marker for this key trait will be of great value to blackcurrant breeders, permitting progeny selection for this trait at a much earlier stage in the breeding process resulting in a much lower labour requirement. The work presented here reveals the identification of a potential marker for blackcurrant AsA content in that three genotypes with high AsA levels all showed the same point mutations in the gene encoding GDPME. These genotypes were not closely related having the parents 'Ben Lomond' x 'Polar', (C2-1-62 x 'Stor Klas') x B1834-75 and (C2-1-62 x 'Ben Alder') x B1834-75 for genotypes 8992-11, 8982-6 and 8944-13, respectively. Clearly significant further work needs to be undertaken in order to confirm the utility of the potential marker and this is currently ongoing within the SCRI breeding programme.

CONCLUSIONS

Previous work has shown that AsA accumulation occurs primarily as a result of *in situ* biosynthesis within the fruit via the L-galactose pathway and that accumulation ceases as a result of decreased biosynthesis and increased turnover (Hancock *et al.* 2007). In the present work the impact of both genotype and environment on fruit AsA content is highlighted and it is shown that while prevailing environmental conditions can have a significant impact on the AsA content of fruit, underlying genetic factors also have a role with phenotypic hierarchy generally maintained year-to-year. These data suggest that breeding for AsA content in blackcurrant is an achievable aim.

Analysis of expression of genes encoding four key enzymes of the AsA biosynthetic pathway failed to show correlation either between AsA biosynthetic capacity at different fruit developmental stages within a single genotype or ripe fruit AsA content across genotypes suggesting that control of AsA biosynthesis within blackcurrant fruit lies within post-transcriptional and/or post-translational mechanisms.

Sequence analysis of the gene encoding GDPME revealed considerable diversity between genotypes, with three high AsA yielding genotypes showing identical polymorphisms despite genetically diverse parentage. Such polymorphism has the potential to act as a predictive marker for fruit vitamin C content and further studies will be directed towards validation and mapping of these markers.

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