Ascorbic Acid-Related Genes

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Abstract

The fruit of *Actinidia* are unusual in that they contain very high concentrations of vitamin C—over 80 mg/100 g fresh weight (FW) in commercial cultivars and over 800 mg/100 g FW in some wild species. In this review, we describe the genes for various proposed pathways for ascorbate production, via L-galactose, via glucuronate from myo-inositol and via galacturonate from pectin. We then focus on the L-galactose pathway genes and enzymes identified in kiwifruit. We also discuss the presence of genes that recycle ascorbate and the production of oxalate, another metabolite with a high concentration in kiwifruit. Lastly, we discuss two levels of regulation of ascorbate biosynthesis in kiwifruit, at the transcriptional level through the gene that encodes the enzyme GDP-galactose phosphorylase (GGP) and at the translational level through feedback control of *GGP* translation involving a upstream open reading frame on the 5′ untranslated region of *GGP*.

13.1 Introduction

Vitamin C or ascorbate is not synthesised by humans and is consequently needed in the diet with the best dietary sources of ascorbate being green vegetables and fruits. Of the commercially significant and traded dessert fruit, kiwifruit has the highest concentration of vitamin C. While other fruit have higher ascorbate (e.g. acerola), they are either not significantly traded or mainly processed (frozen or juiced, e.g. blackcurrants and dried, e.g. acerola). *Actinidia chinensis* var. *deliciosa* 'Hayward' kiwifruit has 85 mg ascorbate/100 g fresh weight (FW); *Actinidia chinensis* var. *chinensis* 'Hort16A' marketed as Zespri® Gold Kiwifruit has 105–110 mg ascorbate/100 g FW while *Actinidia eriantha* can have over 800 mg/100 g FW. On a fresh weight basis, green 'Hayward' and gold 'Hort16A' kiwifruit contain 50 % more vitamin C than an orange, five or six times as much as a banana or

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ten times as much as an apple (Huang and Ferguson 2007). High vitamin C concentration is one of the reasons that kiwifruit are rated as very healthy (Ferguson and Ferguson 2003).

There is considerable variation between *Actinidia* species in ascorbate concentration, from low values in fruit of *Actinidia henryi* (4.4 mg/100 g FW) and *Actinidia rudis* (5) to very high in *Actinidia latifolia* (671–2140) and *A. eriantha* (500–1379 mg/100 g FW) (Huang et al. 2004). Fruit of *Actinidia kolomikta* also contain high amounts of ascorbate (650–850) (Chesoniene et al. 2004). When the total amount of vitamin C concentration per fruit is calculated, the best sources are the fruit of *A. eriantha*, *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa*, all of which have larger fruit. There is also large variation in the vitamin C concentration within a species (Huang and Ferguson 2007). Accessions of *A. chinensis* var. *chinensis* range from 50 to 420 mg ascorbate/100 g FW (Huang et al. 2004) while 143 fruiting plants from a single cross of *A. chinensis* var. *chinensis* ranged from 49 to 209 mg/100 g FW [A.R. Ferguson, unpublished, (Bulley et al. 2009)]. Values for ascorbate concentrations in fruit of accessions of *A. chinensis* var. *deliciosa* range from 30 to ca. 400 mg/100 g FW (Ferguson 1991). While the levels of the acids citrate, quinate and malate are usually much higher than ascorbate (Marsh et al. 2009) in cultivars of *A. chinensis* var. *chinensis, A. chinensis* var. *deliciosa* and *Actinidia arguta*, this is not the case in ultra-high ascorbate *Actinidia* genotypes.

In addition, in some *Actinidia* species, ascorbate content has been shown to have a high heritability (Cheng et al. 2004) which is linked to increased soluble sugar content. In the *A. chinensis* var. *chinensis* cross mentioned above, a significant QTL for ascorbate was detected in the 143 progeny (M.A. McNeilage et al. personal communication) on the genetic map (Fraser et al. 2009). In segregating back-cross populations (*A. eriantha* × *A. chinensis* var. *chinensis* or *A. chinensis* var. *deliciosa* back-crossed to either *A. chinensis* var. *deliciosa* or *A. chinensis* var. *chinensis*), a simple genetic model with two main loci will fit the measured ascorbate data (W.A. Laing and A.G. Seal unpublished).

13.2 Biochemical Pathways of Ascorbate Biosynthesis

One main pathway of ascorbate biosynthesis has been fully documented in various species of higher plants, named the L-Galactose pathway (Wheeler et al. 1998; Linster and Clarke 2008; Wheeler et al. 2015), starting from glucose, and all the genes in the pathway have been identified (Fig. 13.1) and shown to have activity as proteins (including kiwifruit, W.A. Laing et al. unpublished). The genes for this pathway have been identified, cloned and validated from various *Actinidia* species (Laing et al. 2004a, 2007; Richardson et al. 2004; Linster et al. 2008; Bulley et al. 2009; Torabinejad et al. 2009; Li et al. 2011; Bulley et al. 2012; Li et al. 2013a, b, c, 2014). Alternative pathways through galacturonate and glucuronate (Li et al. 2010b) have been proposed but not all the genes and enzymes have been identified. The glucuronate pathway could either derive from glucose via UDP-glucose or from conversion of myo-inositol conversion to glucuronate by myo-inositol oxygenase. The most likely precursor to the galacturonate pathway would be either pectin or UDP-glucuronate epimerisation to UDP-galacturonate. However, polygalacturonase gene expression (Wang et al. 2000) and kiwifruit cell wall breakdown occurs later in fruit development and ripening when ascorbate concentrations are stable (Bulley et al. 2009).

13.2.1 L-Galactose Pathway

13.2.1.1 Conversion of Glucose-6-P to Gannose-1-P

The genes encoding the enzymes for these conversions have all been identified in kiwifruit and do not appear to be limiting ascorbate formation, although overexpression of phosphomannomutase (*PMM*) has been shown to increase ascorbate in leaves of other species (Qian et al. 2007; Badejo et al. 2009). It is possible that, under some circumstances, carbon supply to ascorbate biosynthesis might limit ascorbate synthesis. Two phosphomannose isomerase (*PMI*) genes exist in *Arabidopsis* with *PMI1* being shown to

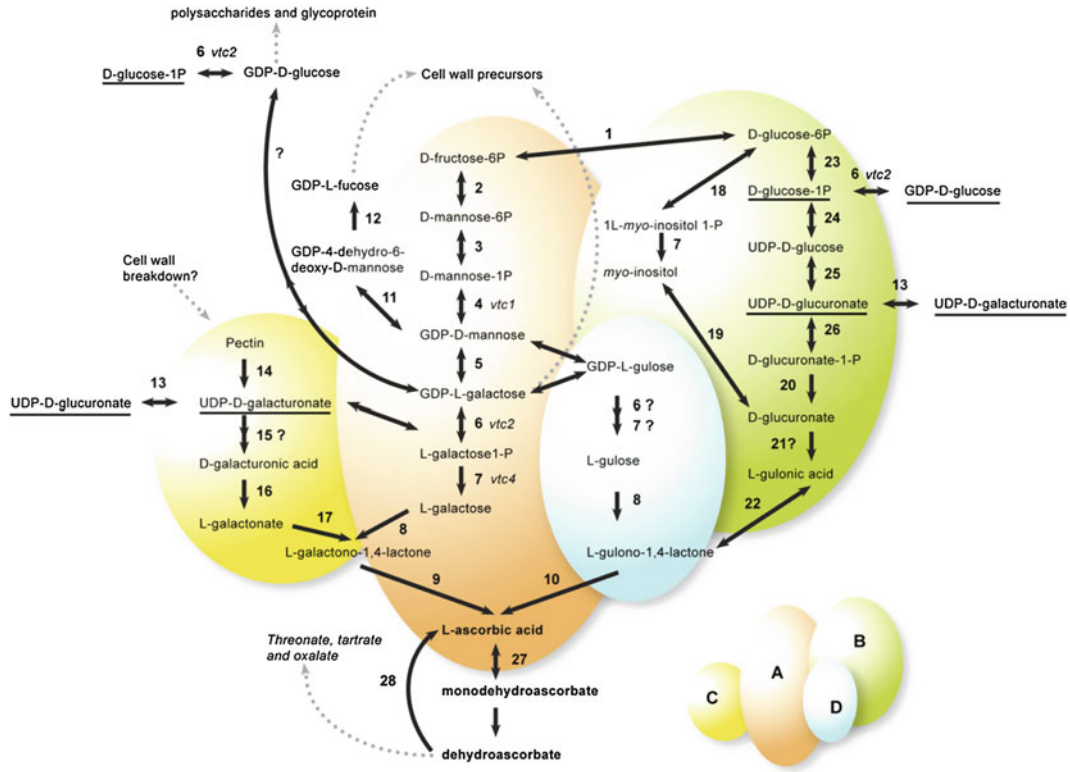

Fig. 13.1 Reactions, enzymes and context of ascorbic acid biosynthesis and regeneration in plants. (*A*) L-Galactose pathway, reactions *2*–*9*. (*B*) myo-Inositol/glucuronate pathway, reactions *7*, *18*–*26*. (*C*) Galacturonate pathway, reactions *14*–*17*. (*D*) L-Gulose pathway, possible reactions *5*, *6*, *7*, *8* and *10*. Reactions with question marks after the number are hypothetical and the exact enzyme is yet to be identified. Underlined chemical names are those that appear in more than one position in the diagram. Gene expression of transcripts of numbered enzymes in bold type was analysed. *1*, glucose-6-phosphate isomerase; *2*, mannose-6-phosphate isomerase; *3*, phosphomannomutase; *4*, GDP-mannose pyrophosphorylase; *5*, GDP-mannose-3′,5′-epimerase; *6*, GDP-L-galactose transferase; *7*, L-galactose-1-phosphate phosphatase; *8*, L-galactose dehydrogenase; *9*, L-galactono-1,4-lactone dehydrogenase; *10*, L-gulono-1,4-lactone oxidase; *11*, GDP-D-mannose-4,6-dehydratase; *12*, GDP-L-fucose synthase; *13*, UDP-galacturonate epimerase; *14*, polygalacturonate 4-α-galacturonosyltransferase; *15*, galacturonate-1-phosphate uridylyltransferase and galacturonate-1-phosphate phosphatase (hypothetical); *16*, D-galacturonic acid reductase; *17*, aldonolactonase; *18*, L-myo-inositol 1-phosphate synthase; *19*, myo-inositol oxygenase; *20*, D-glucurono-1-phosphate phosphatase; *21*, glucuronate reductase; *22*, gulonolactonase; *23*, phosphoglucomutase; *24*, UDP-glucose-pyrophosphorylase; *25*, UDP-glucose dehydrogenase; *26*, glucuronate-1-phosphate uridylyltransferase; *27*, monodehydroascorbate reductase; *28*, dehydroascorbate reductase; vtc, vitamin C content (Bulley et al. 2009. With the permission of Oxford University Press)

be essential for ascorbate synthesis and the only one to be expressed constitutively, with *PMI2* not being expressed in light (Maruta et al. 2008). Both *PMI* isoforms were feedback inhibited by ascorbate showing a higher level general cut-off mechanism to be in place before metabolism enters ascorbate biosynthesis proper (Maruta et al. 2008).

Phosphoglucoisomerase (*PGI*), *PMI* and *PMM* are all represented in the kiwifruit genome (Huang et al. 2013) and found in the kiwifruit EST collection (Crowhurst et al. 2008) (Table 13.1).

Table 13.1 Ascorbate-related genes in kiwifruit. BLASTp searches based on identified or postulated *Arabidopsis* peptide sequences were run against *Actinidia chinensis* var. *chinensis*-predicted protein sequences (Huang et al. 2013)

Ref	Enzyme name	Abbreviation	Candidate *Arabidopsis* homologues	Kiwifruit ID
1	Phosphoglucoisomerase	PGI	At5g42740 [At4g24620]	Achn197361, Achn014461 Achn087691, Achn221981
2	Phosphomannose Isomerase	PMI	At1g67070	None
			At3g02570	None
3	Phosphomannomutase	PMM	At2g45790	Achn302501[a]
4	GDP-mannose pyrophosphorylase	GMP	AT2G39770 (VTC1)	Achn212141, Achn258021, Achn055281
			AT3G55590	Achn212141, Achn258021, Achn055281
			AT4G30570	Achn212141, Achn258021, Achn055281
			AT1G74910	Achn116891, Achn198001, Achn137621, Achn323951
5	GDP-mannose-3′,5′-epimerase	GME	At5g28840	Achn054171, Achn030021
6	GDP-L-galactose phosphorylase	GGP	At4g26850 (VTC2)	Achn155031, Achn339231
			AT5G55120 (VTC5)	Achn155031, Achn339231
7	L-Galactose-1-phosphate phosphatase	GPP	At3g02870 (VTC4)	Achn341571[b]
8	L-Galactose dehydrogenase	GalDH	At4g33670	Achn334011
9	L-Galactono-1,4-lactone dehydrogenase	GalLDH	At3g47930	Achn136491
10	L-Gulono-1,4-lactone oxidase/D-Arabinino-1,4-lactone oxidase	GuLO	**At2g46740**	Achn346051
			At2g46750	Achn346051
			At2g46760	Achn346051
			At1g32300	Achn346051
			At5g11540	Achn346051, Achn368611
			At5g56490	Achn346051
11	GDP-D-mannose-4,6-dehydratase	MUR	At5g66280	Achn207151, Achn053271, Achn053211
			At3g51160	Achn207151, Achn053211, Achn053271
12	GDP-L-fucose synthase/GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase	GER	At1g17890	Achn256791
			At1g73250	Achn256791
	Fucose-1-phosphate guanylyltransferase/fucokinase		At1g01220	Achn104351, Achn136001
13	UDP- glucuronate epimerase/UDP-galacturonate epimerase	UGalE/UGluE/GAE	At4g30440	Achn092741
			At1g02000	Achn092741
			At4g00110	Achn092741
			At2g45310	Achn092741
			At4g12250	Achn092741
			At3g23820	Achn092741
14	Polygalacturonate 4-α-galacturonosyltransferase	PGT	Numerous	
15	Galacturonate-1-phosphate uridylyltransferase and Galacturonate-1-phosphate phosphatase (hypothetical)	GalUT/GalPP	No identified candidates	

(continued)

Table 13.1 (continued)

Ref	Enzyme name	Abbreviation	Candidate *Arabidopsis* homologues	Kiwifruit ID
16	D-Galacturonic acid reductase	GalR	At1g59960	Achn144491, Achn162311, Achn105301, Achn073761, Achn022001
			At1g59950	Achn144491, Achn162311, Achn022001, Achn105301, Achn073761
			At2g37790	Achn375871
17	Aldonolactonase	AL	No identified candidates	
18	L-Myo-inositol 1-phosphate synthase	MIPS	At2g22240	Achn171511, Achn332511, Achn093941[c]
			At4g39800	Achn171511, Achn332511, Achn093941[d]
			At5g10170	Achn171511, Achn332511, Achn093941[d]
19	Myo-inositol oxygenase	MIOX	At4g26260	Achn310731, Achn216311, Achn130711, Achn310751, Achn050681
			At1g14520	Achn310731, Achn216311, Achn130711, Achn310751, Achn050681
			At2g19800	Achn310731, Achn216311, Achn130711, Achn310751, Achn050681
			At5g56640	Achn310731, Achn130711, Achn216311, Achn310751, Achn050681
20	D-Glucurono-1-phosphate phosphatase	GluPP	No identified candidates	
21	Glucuronate reductase	GluR	No identified candidates	
22	Gulonolactonase	GuL	No identified candidates	
23	Phosphoglucomutase	PGM	At1g23190	Achn284661, Achn285641
			At1g70730 [At5g51820]	Achn284661, Achn285641 Achn284661, Achn285641
24	UDP-glucose-pyrophosphorylase	UGP	At5g17310	Achn388541, Achn239081, Achn191791, Achn171071
			At3g03250	Achn388541, Achn239081, Achn191791, Achn171071
25	UDP-glucose dehydrogenase	UGD	At3g29360	Achn194861, Achn390661, Achn335381, Achn256641, Achn161931, Achn335371
			At5g39320	Achn390661, Achn194861, Achn335381, Achn256641, Achn161931, Achn335371
			At5g15490	Achn194861, Achn390661, Achn335381, Achn256641, Achn161931, Achn335371
			At1g26570	Achn194861, Achn390661, Achn335381, Achn256641, Achn161931, Achn335371

(continued)

Table 13.1 (continued)

Ref	Enzyme name	Abbreviation	Candidate *Arabidopsis* homologues	Kiwifruit ID
26	Glucuronate-1-phosphate uridylyltransferase	GluPU	At5g52560	Achn029421
27	Monodehydroascorbate reductase	MDAR	At1g63940	Achn005611, Achn132811, Achn091771
			At3g09940	Achn091771, Achn075231, Achn389481, Achn297231
			At3g27820	Achn389481, Achn297231, Achn091771, Achn075231
			At3g52880	Achn091771, Achn075231, Achn389481, Achn297231
			At5g03630	Achn091771, Achn075231, Achn389481, Achn297231
28	Dehydroascorbate reductase	DHAR	At1g19570	Achn278191[e], Achn224431[f]
			At1g75270	Achn278191[g], Achn224431[h]
			At5g16710	Achn278191, Achn224431
Regulatory proteins				
	Protein kinase/protein phosphatase	VTC3	At2g40860	Achn270901
	Ethylene response factor subfamily b-3 of ERF/AP2 transcription factor family	AtERF98	At3g23230	Achn246791[i]
	Ascorbic acid mannose pathway regulator 1 (F box protein)	AMR1	At1g65770	None
	Cop9-signalosome 5b	CSN5B	At1g71230	Achn105101
	Constitutive photomorphogenic 9	CSN8	At4g14110	Achn175751
Transporter				
	Anion transporter 2	ATPHT4;4	At4g00370	Achn302711

Ref is number of the enzymes shown in Fig. 13.1
In blast searches of *Arabidopsis* peptide sequences on *A. chinensis* var. *chinensis* 'Hongyang'-predicted proteins (Huang et al. 2013), only matches with $E < e{-}100$ were listed except where noted ([a]E = 5e–80; [b]E = 3e–44; [c]E = 3e–98; [d]E = 2e–97, [e]E = 1e-88; [f]E = 2e–75; [g]E = 1e–92; [h]E = 2e-79; and [i]E = 6e–35). Underlined bold *Arabidopsis* names are the most likely candidates. Genes in brackets are the chloroplast located. Entries with no *Arabidopsis* genes are unidentified in *Arabidopsis*

13.2.1.2 Conversion of Mannose-1-P to Galactose-1-P

GDP-mannose pyrophosphorylase (*GMP*) converts mannose-1-P to GDP-mannose. GDP-mannose provides carbon both for ascorbate synthesis and for cell wall components and protein modification (Zablackis et al. 1996; Keller et al. 1999; Handford et al. 2003). In some circumstances, it is probable that the supply of carbon skeletons may limit ascorbate biosynthesis which may be why transformation with the *GMP* gene has variable effects of ascorbate concentration (Badejo et al. 2007; Wang et al. 2011; Cronje et al. 2012; Imai et al. 2012; Zhou et al. 2012; Zhang et al. 2015a). It is possible that upstream *PMI* expression levels may also be limiting and this suggests that coexpression of PMI might overcome such limitations.

GDP-mannose epimerase (*GME*) is another key gene in that it results in the formation of GDP-L-galactose which is almost all used for ascorbate production, with little L-galactose being found elsewhere in the metabolome (Gilbert et al. 2009). However, by itself *GME* has little effect of ascorbate concentrations in plant tissues (see below).

GDP-galactose phosphorylase (*GGP*) which converts GDP-galactose to galactose-1-phosphate is the key enzyme in the ascorbate pathway and has been shown to control ascorbate in a wide range of

species (Laing et al. 2007; Bulley et al. 2009, 2012; Zhang et al. 2011, 2015a; Zhou et al. 2012; Li et al. 2013a, b, c; Huang et al. 2014; Ma et al. 2014). The evidence is either from the studying patterns of gene expression of these genes during fruit development in kiwifruit or from the over-expression of kiwifruit genes in other species.

Transformation with *GGP* results in very significant increases in tissue ascorbate concentrations (Laing et al. 2007; Bulley et al. 2012; Zhou et al. 2012; Zhang et al. 2015a). While transformation with GME alone has small effects on ascorbate in a range of species (Zhang et al. 2011, 2015b; Huang et al. 2014; Ma et al. 2014), coexpression of *GME* and *GGP* results in a strong synergistic increase on ascorbate concentration (Bulley et al. 2009; Laing et al. 2015). *GME* gene transcription also tracks ascorbate production along with GGP transcription (Bulley et al. 2009; Li et al. 2013c).

13.2.1.3 Conversion of Galactose-1-P to Ascorbate

Galactose-1-P phosphatase (*GPP*) (Laing et al. 2004b), galactose dehydrogenase (*GalDH*) (Laing et al. 2004a; Li et al. 2010b) and galactono lactone dehydrogenase (*GalLDH*) (W.A. Laing unpublished) have all been identified in kiwifruit, but again do not appear to regulate ascorbate formation, except when activity is reduced (Tabata et al. 2001; Gatzek et al. 2002; Conklin et al. 2006; Alhagdow et al. 2007; Imai et al. 2009; Torabinejad et al. 2009; Zhou et al. 2012; Zhang et al. 2015b). However, it has been suggested *GPP* may be involved in light and abiotic stress responses (Li et al. 2013b). In tomato, *GPP* has been suggested to play an important role in regulating ascorbate accumulation during fruit development (Ioannidi et al. 2009), and while its expression was the highest of the L-galactose pathway genes, *GME* and more particularly *GGP* expression also correlated well with ascorbate accumulation. A later study, also in tomato, found that 'translocation from source leaves and biosynthesis via the D-mannose/L-galactose pathway are dominant sources in immature fruits, while the alternative D-galacturonate pathway contributes to AsA [ascorbate] accumulation in ripened Micro-Tom fruits' (Badejo et al. 2012). It therefore appears that a possible explanation for the very high expression of GPP in ripening tomato fruit may be it is there to convert the L-galactose-1P postulated to be derived from the D-galacturonate pathway via the conversion of UDP-D-galacturonate to L-galactose-1P (Fig. 13.1).

13.2.2 L-Glucuronate Pathway and Myo-Inositol

A. chinensis var. *deliciosa* and *A. arguta* contain high concentrations of myo-inositol in the leaves and fruit (Bieleski et al. 1997; Klages et al. 1998) which potentially could serve as a substrate of ascorbate production by conversion of myo-inositol to glucuronate by myo-inositol oxygenase. Myo-inositol-1-P is synthesised from glucose-6-P by myo-inositol synthase and then, myo-inositol-1-P is dephosphorylated by the enzyme myo-inositol phosphatase (Gillaspy et al. 1995). This latter enzyme is the same as that encoded by *GPP*, L-galactose-1-P phosphatase (Laing et al. 2004b) in both kiwifruit and *Arabidopsis* (Torabinejad et al. 2009). Good homologues of the oxygenase and the synthase have also been identified in a kiwifruit EST collection (Crowhurst et al. 2008). The glucuronate is then converted to L-gulonate by an unknown enzyme, then to the gulonate 1,4 lactone and thence to ascorbate, again by enzymes encoded by unknown genes. However, possible candidates for these genes are found in the kiwifruit EST database (Crowhurst et al. 2008) although they are not validated, even in other species. The other genes that encode enzymes that convert glucose to glucuronate (UDP-glucose dehydrogenase, glucuronate-1-P uridyltransferase and glucurono-1-P phosphatase) are validated in other species and good homologues are present in the kiwifruit genome and EST databases.

13.2.3 L-Galacturonate Pathway

This pathway is proposed to result in ascorbate production from the breakdown of cell wall pectin into galacturonate (Di Matteo et al. 2010)

or from the conversion of UDP-glucuronate into UDP-galacturonate by UDP-glucuronate epimerase (Gu and Bar-Peled 2004; Usadel et al. 2004). The UDP-galacturonate is then hydrolysed to galacturonate (this requires a uridylyltransferase and a phosphatase, no enzymes characterised) and then reduced to galactonate by galacturonate reductase (Agius et al. 2003). A homologue to the validated strawberry galacturonate reductase is found in kiwifruit ESTs (Crowhurst et al. 2008) and in the kiwifruit genome (Huang et al. 2013) with 65–67 % identity and 83 % similarity, respectively. The kiwifruit gene has been cloned and expressed in *Escherichia coli*, but it showed no reductase activity (Li et al. 2010b, 2011). While the strawberry gene has been shown to increase ascorbate in tomato (Amaya et al. 2014), potato (Hemavathi et al. 2009) and *Arabidopsis* (Agius et al. 2003), this gene is not thought to control ascorbate in kiwifruit (Li et al. 2010b, 2011).

13.3 Ascorbate Recycling Enzymes

A reactive oxygen species or an oxidising agent such as H_2O_2 oxidises ascorbate to monodehydroascorbate (MDHA), which is either directly reduced back to ascorbate by monodehydroascorbate reductase (MDHAR) or non-enzymatically disproportionated into ascorbate and dehydroascorbate (DHA). DHA is then reduced back to ascorbate through reducing equivalents provided by glutathione either chemically or by dehydroascorbate reductase (DHAR). Oxidised glutathione is finally reduced by glutathione reductase. This redox hub maintains the ascorbate in a mostly reduced state (Foyer and Noctor 2011). There are five MDHAR genes and three DHAR genes in *Arabidopsis*.

Different studies have overexpressed *DHAR* (Chen et al. 2003; Kwon et al. 2003; Eltayeb et al. 2006; Goo et al. 2008; Naqvi et al. 2009; Yin et al. 2010; Haroldsen et al. 2011; Qin et al. 2011; Huang et al. 2014) and *MDHAR* (Kavitha et al. 2010; Li et al. 2010a; Yin et al. 2010; Haroldsen et al. 2011; Gest et al. 2012) in a range of plants and, except for maize kernels (only very low ascorbate is present in seeds), the concentration of ascorbate was mostly little affected. None of these studies was performed in kiwifruit. However, many papers reported an increase in the ratio of reduced to oxidised ascorbate and an improvement in stress resistance.

Various versions of the two ascorbate recycling genes are found in the kiwifruit genome (Table 13.1). However, there appears to be no published work on *MDHAR* and *DHAR* genes in kiwifruit. We have constitutively overexpressed a kiwifruit *DHAR* in *Arabidopsis* which resulted in changes to the ratio of reduced/oxidised ascorbate and also appeared to increase salinity tolerance (S.M. Bulley, unpublished).

13.4 Oxalate in Kiwifruit

Kiwifruit fruit also contain high amounts of oxalate, much in crystalline (raphide) form (Rassam and Laing 2005; Rassam et al. 2007). In other species, it has been proposed that ascorbate breaks down into oxalate (Keates et al. 2000; Kostman et al. 2001) although little is known about genes involved in the pathway (Green and Fry 2005). The relationship between the amount of oxalate and ascorbate in a plant tissue is not straightforward, suggesting that the breakdown of ascorbate is a regulated process (Rassam and Laing 2005; Rassam et al. 2007).

13.5 Regulation of Ascorbate Biosynthesis

13.5.1 Regulation Through Gene Expression of Pathway Genes

There have been several studies of expression of vitamin C-related genes in kiwifruit (Bulley et al. 2009; Li et al. 2010b, 2013a, c, 2014) and these have shown changes during fruit development and between kiwifruit species and accessions. For example, Bulley et al. 2009 showed large differences during fruit development with a peak in

ascorbate accumulation around four to seven weeks after anthesis and gene expression maxima around the same time. However, the biggest differences between taxa (*A. chinensis* var. *chinensis, A. chinensis* var. *deliciosa* and *A. eriantha*) were for *GGP* and *GME*, and to some extent for *GMP*, with the high ascorbate *A. eriantha* having much higher expression levels of these genes. Through transient and stable transformation of *Arabidopsis thaliana* and *Nicotiana benthamiana* with kiwifruit genes, it was concluded that *GGP* and *GME* synergistically controlled ascorbate biosynthesis. Other results (Li et al. 2014) also supported the difference in *GGP* expression between *A. eriantha* (high ascorbate) and *Actinidia rufa* (low ascorbate). On the other hand, Li et al. 2010b showed a similar pattern of ascorbate accumulation and gene expression during fruit development, for the one cultivar *A. chinensis* var. *deliciosa* 'Qinmei'. Based on correlations between gene expression and ascorbate concentrations, they concluded *GPP* controlled ascorbate biosynthesis. Various light and stress conditions have also been shown to affect gene expression of *GGP*, *GME* (Li et al. 2013c) and *GPP* (Li et al. 2013b) in kiwifruit, but these studies do not determine where the control of ascorbate biosynthesis lies in fruit. Correlations between gene expression and a metabolite support but do not prove that a gene product regulates the metabolite, and it is likely some degree of coordination between genes in a pathway would occur. Some recent sequencing of an *A. chinensis* var. *chinensis* kiwifruit during fruit development is shown in Fig. 13.2. These data (L. Luo et al. unpublished) are consistent with other published studies mentioned above.

Various studies have established *GGP* as a key regulator of ascorbate biosynthesis in many plants, and most work has been done on this gene (Laing et al. 2007; Bulley et al. 2009; Li et al. 2013a, b, 2014). This is reasonable given that the GGP enzyme catalyses the first committed step in ascorbate biosynthesis, although others have suggested *GMP* as the key control gene (Wang et al. 2013a). Gene expression of *GGP* and *GME* in a range of species is strongly regulated by light (Dowdle et al. 2007; Yabuta et al. 2007; Gao et al. 2011; Massot et al. 2012) and abiotic stress (Li et al. 2013a), both factors regulate ascorbate concentrations. In addition, as discussed above, expression of these two genes relates to the ascorbate concentrations in different kiwifruit species with different ascorbate concentrations (Bulley et al. 2009; Li et al. 2014). More significantly, transient transformation of *Nicotiana benthamiana* with various kiwifruit genes from the ascorbate biosynthetic pathway shows that *GGP* strongly affects ascorbate, and *GME* has little effect while a combination of *GME* and *GGP* synergistically stimulates ascorbate concentration (Bulley et al. 2009, 2012; Laing et al. 2015). Other genes in the pathway except *GMP* have little effect on ascorbate concentration ((Zhou et al. 2012), W.A. Laing unpublished). In addition, genetic mapping in apple has established that the QTLs for ascorbate colocate with *GGP* orthlogs (Mellidou et al. 2012) and that transformation of strawberry, potato and tomato with kiwifruit or *Arabidopsis GGP* results in significantly increased ascorbate (Bulley et al. 2012). *GGP* gene expression also shows a strong diurnal trend, peaking during the morning and falling during the day in *Arabidopsis* (Dowdle et al. 2007), suggesting that ascorbate biosynthesis potential is primed for the period of the day when peak ascorbate is required, at midday maximum light intensity. We have also observed similar diurnal trends in gene expression in the leaves of *Arabidopsis* (W.A. Laing unpublished).

13.5.2 Regulation Through Translation of GGP

It appears well established that transcriptional regulation of the key genes *GGP* and *GME* controls ascorbate concentrations. However, translational regulation of ascorbate concentrations also plays a significant part. Recently, it was shown that the 5′ untranslated region (UTR) of *GGP* from a wide range of species including kiwifruit contains a highly conserved upstream open reading frame (uORF) (Laing et al. 2015). This uORF is unusual in that it starts with a

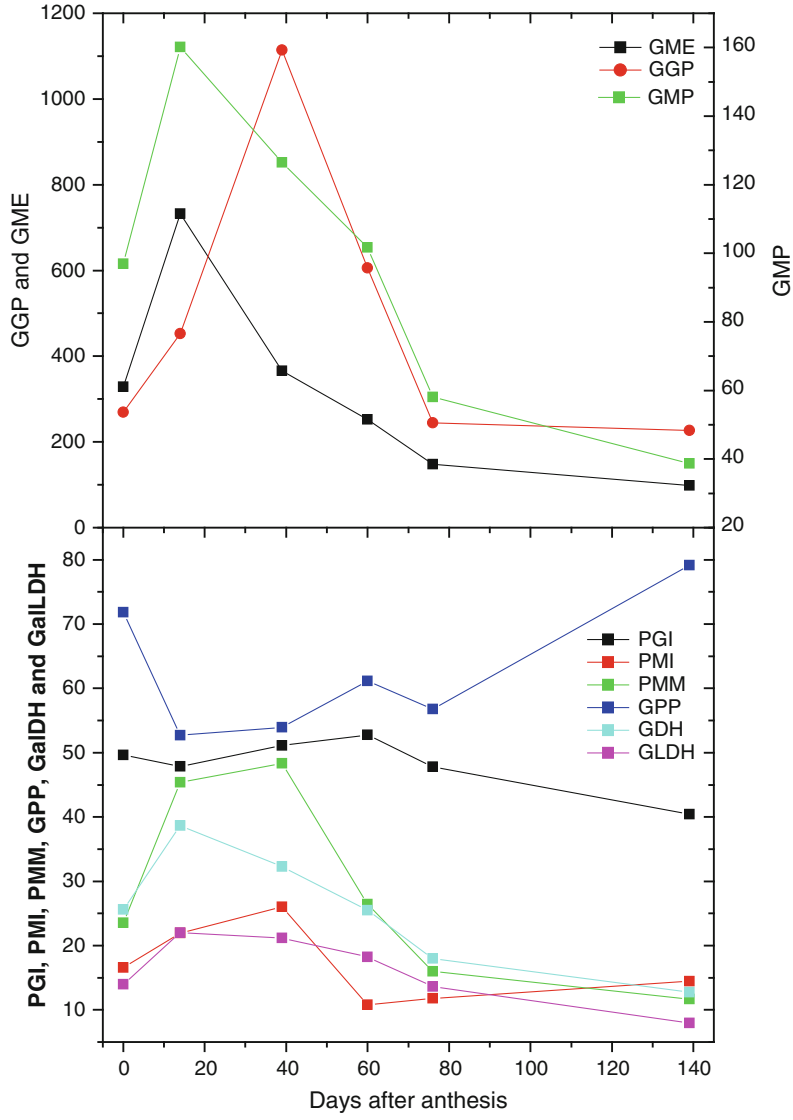

Fig. 13.2 Next-generation sequencing study of gene expression during fruit development in an *Actinidia chinensis* var. chinensis genotype. Data unpublished from L. Luo et al.

non-canonical start codon, ACG. The model proposed was that under high ascorbate concentrations, the uORF is translated and acts as an inhibitor of *GGP* translation. Under low ascorbate, the uORF is skipped and *GGP* is translated. This model provides a direct link between the ascorbate concentration and the production of GGP, the key regulatory enzyme in ascorbate biosynthesis, and allows rapid and feedback responsive control of ascorbate biosynthesis under demand conditions (e.g. high light and low temperatures).

13.5.3 Other Ascorbate Affecting Genes

Four genes that regulate ascorbate in *Arabidopsis* have been identified and characterised. These are a protein kinase/phosphatase (*VTC3*) (Conklin et al. 2013), a transcription factor (*AtERF98*) (Zhang et al. 2012), photomorphogenic factor COP9 signalosome subunit 5B (*CSN5B*) (Wang et al. 2013b) and an F box protein (*AMR1*) (Zhang et al. 2009). *Actinidia* homologues of the first three genes have been found but not of the F

box protein. Little is known how *VTC3* regulates ascorbate except that it is constitutively expressed and does not vary much under a wide range of conditions, suggesting that regulation may be at a post-transcriptional level (Conklin et al. 2013). It is possible that *VTC3* may be a factor in the uORF regulation described above (Laing et al. 2015). *AMR1* appears to be a negative regulator of ascorbate and a negative regulator of the L-galactose pathway of ascorbate biosynthesis genes (Zhang et al. 2009). Inspection of this paper shows that *GGP* and *GME* are the genes most affected by *AMR1*. An obvious hypothesis is that AMR1 targets for degradation a transcription factor that increases transcription of the ascorbate genes. The AtERF98 transcription factor increases the transcription of many of the genes in the L-galactose pathway, GMP especially and GGP (Zhang et al. 2012) and binds with the promoter of *GMP*. However, the authors did not explore how *AtERF98* interacted with other promoters or whether it was a target for *AMR1*. Lastly, CSN5B was shown to interact with the N terminus of GMP and target it for degradation (Wang et al. 2013b). CSN5B appears to promote degradation of GMP protein in the dark through ubiquitination and the proteasome. However, the authors did not test whether CSN5B interacted with other genes in the L-galactose pathway. Interestingly, knockout of another *COP9* signalosome subunit, *CSN8*, increased ascorbate even further than a knockout of *CSN5B* (Wang et al. 2013b).

In addition, an ascorbate transporter located in the chloroplast has also been identified (Miyaji et al. 2015) and an *Actinidia* homologue is present. The transporter would serve to transport ascorbate into the chloroplast from its point of synthesis in the mitochondria.

13.6 Conclusions

The ascorbate biosynthetic pathway in kiwifruit is similar to that found in other species. The most likely pathway to provide the great bulk of ascorbate in kiwifruit is the L-Galactose pathway and regulation of ascorbate concentration is through the *GGP* gene and enzyme, with possibly some higher level feedback by ascorbate against PMI1. Ascorbate content plant tissues appear to be mainly the result of variation in transcription of *GGP* (and *GME* in synergy with *GGP*) as well as at the translational level through feedback regulation by ascorbate of the translation of *GGP*. Regulation through GGP makes sense as it the first committed step in the biosynthesis of ascorbate.

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