Ascorbic acid biosynthesis in higher plants and micro-organisms

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Unlike most animals, humans and a small number of other species are unable to synthesise L-ascorbic acid (L-AA; vitamin C) and must obtain the vitamin by dietary intake. As L-AA is the major soluble antioxidant found in higher plant tissues, plant foods represent the primary source of this essential compound in the human diet. Together with flavonoids, polyphenolics and water insoluble compounds such as α-tocopherol (vitamin E), L-AA contributes to the overall intake of 'free radical scavengers' or 'antioxidative metabolites' in the human diet. There is now convincing evidence that such metabolites singly and in combination, benefit health and well-being, acting as anti-cancer forming agents and protecting against coronary heart disease. There is increasing pressure for increasing the recommended daily intake (RDA) of L-AA currently at 60 mg/day in the UK but recently raised to 90 mg in the US. Although, vitamin C intake through synthetic supplements is widespread, it is accepted that intake of vitamin C from foods has the great advantage of simultaneously providing many other health promoting nutrients, such as bioflavonoids, carotenes and iron whose absorption is facilitated by vitamin C.

The goal of developing 'functional foods' or products with enhanced 'nutraceutical' qualities has become a major goal world-wide – not necessarily to prolong life but to ensure a high quality of life. Therefore one objective of our research at SCRI is to optimise the level of L-AA in the edible parts of crop plants based on a sound fundamental knowledge of the genetics, biochemistry and physiology of L-AA biosynthesis and distribution in plants. This strategy may therefore be beneficial not only for the increased quality value of the crop as a commodity but also through increased performance of crop plants in the field. In fact L-AA is also essential for plant life through detoxification of peroxide, ozone, and free radicals and providing protection from damage caused by the accumulation of ROS generated under adverse environmental conditions. Moreover L-AA plays a key role in photosynthesis by detoxifying superoxide anions and hydrogen peroxide in chloroplasts and by regenerating the membrane-soluble antioxidants (α-tocopherol) and zeaxanthin.

In addition to naturally synthesised L-AA, over 80,000 tonnes of synthetic L-AA are produced each year and used to manufacture vitamin supplements, in the food processing and beverage manufacturing industries as well as a supplement in animal feeds. The majority of L-AA is currently produced by primarily chemical means via the seven-step Reichstein process. Alternative methods use prokaryotic fermentations to synthesise intermediates of the Reichstein process, as these industrially useful micro-organisms lack the ability to synthesise L-AA. However, progress in our understanding of L-AA biosynthesis in plants have resulted in the development of alternative strategies for the synthesis of this important commodity with a global market in excess of U.S. $ 600 million.

L-AA Biosynthesis and Distribution in Plants The L-AA biosynthetic pathway in animals was elucidated during the 1950's following experiments with unlabelled and ^{14}C-labelled precursors in rats. Progress in the area of L-AA biosynthesis in plants has been much slower. Early pathways proposed for the biosynthesis of L-AA in higher plants[1] based on the animal model conflicted with biochemical observations from radiolabelling experiments. However, in recent years a novel pathway has been proposed by Smirnoff and coworkers, which fits all the currently available data[2] (Fig. 1). The proposed pathway differs substantially from the animal pathway and involves the synthesis of L-galactose a rare sugar never prior described in plants in its free form. Since its proposition, support for the pathway has been obtained from the study of *Arabidopsis* mutants in which several of the proposed pathway enzymes are down-regulated and that show a substantial decrease in total L-AA content[3]. The biosynthetic pathway identified in *Arabidopsis* has since been confirmed in a wide range of other plants including crop plants such as potato, celery, barley and blackcurrant (Table 1). Evidence of its operation has been obtained from both green tissues and non-photosynthetic storage organs such as fruit, tubers and developing seeds. The current consensus is that this pathway represents the major route for L-AA biosynthesis in higher plants, although the presence of other minor pathways has not been dismissed.

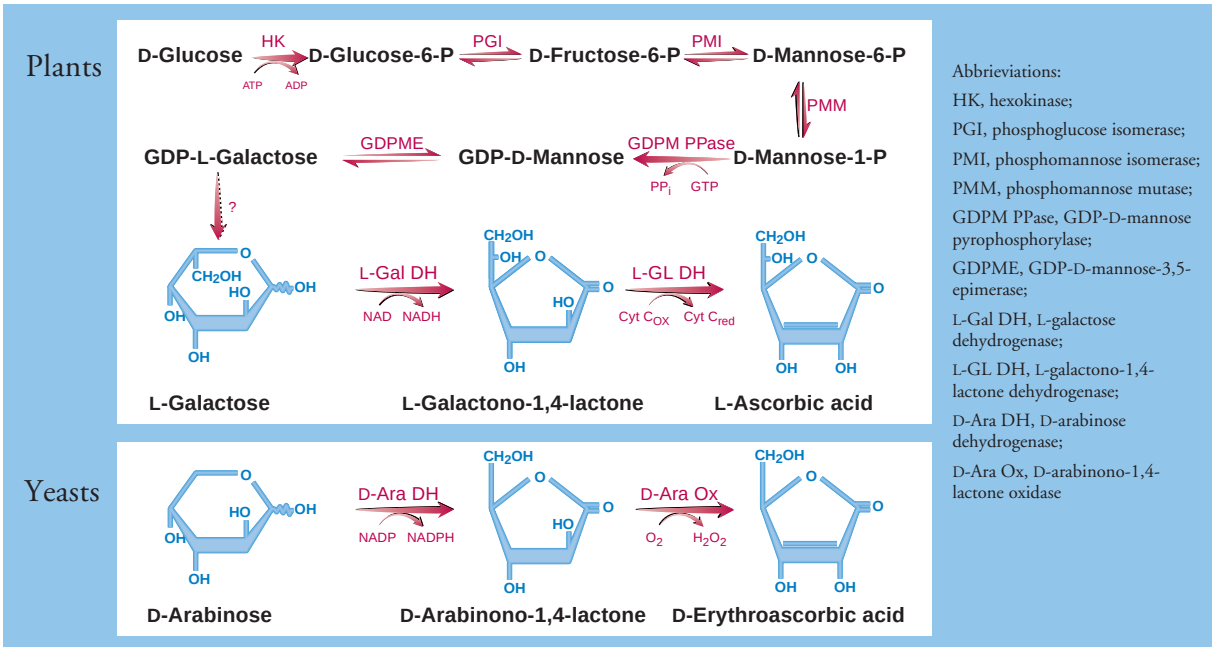

Figure 1 The L-AA Biosynthetic Pathway in Plants and D-EAA Biosynthetic Pathway in Yeasts.
Plants synthesise L-AA from D-glucose via a ten-step pathway involving the sugar nucleotides GDP-D-mannose and GDP-L-galactose. The pathway also utilises the rare sugar L-galactose. Yeasts synthesise the 5-carbon L-AA analogue D-erythroascorbic acid (D-EAA) from the structurally related 5-carbon sugar D-arabinose.

The resolution of the pathway of L-AA biosynthesis has paved the way for the identification of genetic and biochemical factors controlling L-AA accumulation in plants. As a first step to achieving this objective, we undertook biochemical analysis of the pathway using the unicellular chlorophyte *Chlorella pyrenoidosa* as a model system. The advantages of this system included the relative simplicity of the organism compared with multi-cellular and multi-organ higher plants and the availability of L-AA hyperaccumulating lines produced by chemical mutagenesis[4]. For our studies we investigated the two mutant lines H1 and H2, which contained *ca.* 5.1 and 9.8 fold more L-AA than the wild-type respectively. Firstly, we established that both higher plants and *C. pyrenoidosa* cells share a similar pathway as deduced by the pattern of label incorporation into L-AA from radiolabelled substrates (Table 2). In all cases, the observed incorporation of radioactivity into L-AA from each substrate was consistent with its position within the proposed L-AA biosynthetic pathway (see Fig. 1). In the *C. pyrenoidosa* mutants the higher incorporation of D-[U-^{14}C]glucose or D-[U-^{14}C]mannose into L-[^{14}C]AA compared with the wild-type strain confirmed that L-AA biosynthetic capacity is up-regulated. However, both mutant strains showed an equivalent or lower incorporation of L-[1-^{14}C]galactose into L-[^{14}C]AA than the wild-type strain, suggesting that the rate of L-galactose conversion into L-AA is unaffected in the mutants. The only reasonable explanation for these results is that up-regulation of L-AA biosynthesis in *C. pyrenoidosa* mutants occurs as a result of increased L-

Tissue	Substrate		
	D-mannose	L-galactose	L-galactonolactone
Potato tuber	1.29 ± 0.04	3.39 ± 0.45	6.07 ± 0.40
Celery parenchyma	ND	6.04 ± 0.70	1.74 ± 0.16
Celery vascular	ND	11.06 ± 0.79	7.59 ± 1.21
Barley leaves	ND	ND	2.18 ± 0.09
Blackcurrant leaves	1.27 ± 0.04	9.99 ± 0.87	4.48 ± 0.26
Blackcurrant berries	0.70 ± 0.24	1.97 ± 0.34	2.74 ± 0.25

Tissues were incubated for 18 h with agitation in 25 mM D-glucose or the appropriate substrate as described. Samples were extracted in 5% metaphosphoric acid containing 5 mM dithiothreitol and total L-AA content determined by HPLC. Relative values are expressed compared to the L-AA content of tissues incubated with D-glucose ± standard deviation. ND – Not determined

Table 1 Effect of potential precursors on L-AA content of higher plant tissues.

Substrate	% Metabolised label incorporated into L-AA			
	Blackcurrant Leaves	*C. pyrenoidosa* WT	*C. pyrenoidosa* H1	*C. pyrenoidosa* H2
D-[U-^{14}C]glucose	0.60 ± 0.43	0.06 ± 0.01	2.70 ± 0.49	0.57 ± 0.04
D-[U-^{14}C]mannose	9.45 ± 0.06	2.53 ± 0.79	10.05 ± 0.04	6.23 ± 1.64
L-[1-^{14}C]galactose	61.25 ± 9.43	58.68 ± 10.10	59.92 ± 13.78	35.82 ± 7.08

Dark grown *C. pyrenoidosa* cells were incubated with 3 µCi of the appropriate precursor and the same quantity of radioactivity was introduced into blackcurrant leaves via the cut petiole. In both cases, incubation was continued for 4 h prior to extraction of tissue in ice-cold 5% $HClO_3$ containing 10 mM L-AA. Cell debris was removed and supernatant neutralised with K_2CO_3. L-[^{14}C]AA was partially purified on SAX SPE and quantified by HPLC with flow scintillation analysis.

Table 2 Incorporation of ^{14}C-labelled precursors into L-[^{14}C]AA by algae and higher plant tissues.

galactose formation. This hypothesis received confirmation following incubation of *C. pyrenoidosa* with D-glucose, L-galactose or L-galactono-1,4-lactone (Table 3). The mutants accumulated substantially more L-AA compared with the wild-type when incubated with D-glucose but all three *C. pyrenoidosa* strains synthesised similar quantities of L-AA from L-galactose or L-galactono-1,4-lactone. These data support the hypothesis that the mutation is prior to L-galactose on the biosynthetic pathway and results in the biosynthesis of more L-galactose in the hyperaccumulating strains. This hypothesis was confirmed by the finding that both *C. pyrenoidosa* mutant strains contained significantly greater amounts of free L-galactose than the wild-type strain (4.04, 15.36 and 13.91 nmol gDW^{-1} for wild-type, H1 and H2 respectively). Further biochemical investigations in the mutants revealed that the increased L-AA phenotype is correlated with up-regulation of the activity of a novel enzyme involved in L-galactose biosynthesis and work is in progress aimed at the isolation and characterisation of the corresponding gene. Further work is also in progress to characterise the biochemical and molecular regulation of the L-AA biosynthetic pathway using a metabolic engineering approach. Genes coding for enzymes involved in the biosynthetic pathway are being cloned from available sources and expressed transiently in *Nicotiana xanthii* protoplasts to test the effect of the up-regulation of individual enzymes on the L-AA biosynthetic flux.

Although we are close to achieving a clear understanding of the biochemistry and genetics of the biosynthetic pathway at the cellular level, our knowledge of the factors affecting L-AA distribution at the whole plant level and particularly to the storage organs remains poor. Whilst leaf L-AA content is generally high with relatively little variability between herbaceous and woody plants, a huge and unexplained variability is observed in the L-AA content of non-green edible plant tissues (Fig. 2). In fruits, for example, the levels vary from 27 mg gFW^{-1} L-AA (in the camu camu, produced by the low-growing tropical shrub *Mirciaria dubia*) to less than 3 µg gFW^{-1} L-AA (in the medlar produced by *Mespilus germanica*). The variability has no apparent taxonomic reason. For example the medlar is a member of the *Rosaceae* family, to which rose hips also belong and which contain over 4000-fold more L-AA. In addition the L-AA content of food crops is also affected by environmental conditions such as location, weather conditions, mineral fertilisation, growth habit and post-harvest storage, which can result in a reduction of up to 80-90% of the initial L-AA content. One explanation for the variability of L-AA content in plant storage organs is that synthesis of this metabolite in these tissues is controlled much less strictly than in leaves where it fulfils essential and critical functions in photosynthetic metabolism. This suggests that it may be possible to design specific strategies aimed at enhancing the L-AA content of the edible parts of food crops without dras-

Precursor	L-AA Concentration (mmol gDW^{-1})		
	Wild type	H1	H2
D-glucose	4.65 ± 0.49	12.07 ± 1.57	19.40 ± 4.95
D-mannose	0.28 ± 0.09	0.40 ± 0.05	3.38 ± 1.64
D-galactose	97.70 ± 7.88	99.20 ± 12.37	95.64 ± 6.82
L-galactono-1,4-lactone	82.27 ± 12.39	75.65 ± 9.90	79.24 ± 4.08

C. pyrenoidosa was grown to mid-logarithmic phase in the dark at 35°C. Carbon substrate was 5 g L^{-1} D-glucose. Cells were harvested, washed in C-free medium and resuspended with the appropriate carbon substrate (5 g L^{-1}). Incubation was continued for 24 h and the culture divided into two aliquots. One aliquot was used for determination of cell dry weight and the other for L-AA determination by HPLC.

Table 3 Effect of potential precursors on L-AA content of *Chlorella pyrenoidosa* strains.

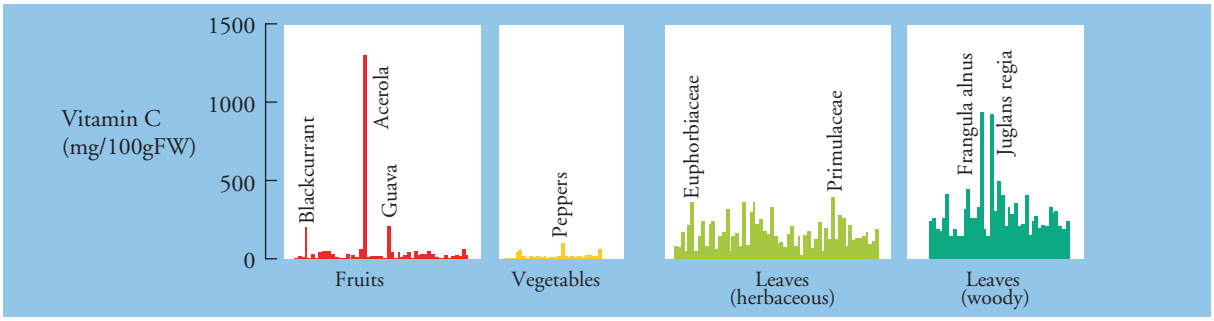

Figure 2 Variability of L-AA Content in Plant Storage Organs and Photosynthetic Tissues
Leaves have a higher average L-AA content than fruits and vegetables, however there is a lower degree of variation in photosynthetic tissues than that in fruits and vegetables. This data suggests some degree of flexibility in the L-AA content of storage organs. One aim of the current research is to understand the factors controlling L-AA concentration in sink tissues and to exploit these mechanisms to enhance the quantity of L-AA in food crops.

tically affecting plant growth or crop performance. Another option is that L-AA is actively transported between plant tissues. We have recently demonstrated the presence of high L-AA concentration in the phloem of many plants including rose, willow, barley and potato. The high concentration of L-AA in the phloem of storage organs (Fig. 3) may be indicative of L-AA transport from L-AA producing sources (leaves?) to non-photosynthetic tissues. We are currently characterising the uptake and transport of L-AA in plant phloem and evaluating the contribution of phloem L-AA on the overall L-AA content of storage organs.

L-AA biosynthesis by micro-organisms At present, the majority of commercially manufactured L-AA is synthesised via the seven step Reichstein process utilising D-glucose as a starting point (Fig. 4). The process involves six chemical steps and one fermentation step for the oxidation of D-sorbitol to L-sorbose. Overall, the yield of L-AA from D-glucose obtained by the Reichstein process stands at ~50%. Although the Reichstein process has all the advantages to be expected after more than 60 years development, it is still highly energy consuming and requires high temperatures and/or pressures for many steps. In addition, most of the chemical transformations involve considerable quantities of organic and inorganic solvents and reagents such as acetone, sulphuric acid and sodium hydroxide. Although some of the compounds can be recycled, stringent environmental control is required, resulting in significant waste disposal costs. These and other economic factors have generated a substantial interest in the industry for the exploitation of microbial biotransformations in the manufacture of L-AA. Yeast, in particular is being considered for this process and our increased understanding of L-AA biosynthesis in plants has provided tools for the development of a novel single-step process for L-AA manufacture

Yeast does not synthesise L-AA but its 5-carbon analogue D-erythroascorbic acid (D-EAA) which has similar antioxidant characteristics to L-AA but it lacks antiscorbutic activity and thus has limited industrial applications. However, the pathway of D-EAA biosynthesis in yeast shares many similarities with the 'committed' steps of the L-AA biosynthetic pathway in plants with the 5-carbon sugar D-arabinose replacing L-galactose (Fig. 1). We tested the specificity of the D-EAA pathway in *S. cerevisiae* by supplying cell cultures

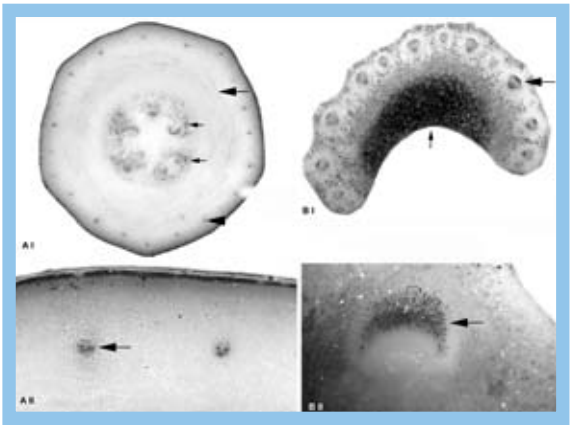

Figure 3 Silver Staining of Plant Storage Organs to Demonstrate Tissue Specific Variability in L-AA Content. Slices of plant tissue were cut and washed briefly. Samples were then stained in an alcoholic silver nitrate solution (pH 2.5) in the dark at 3°C. Excess silver was removed with alcoholic ammonia prior to recording images. In both courgette (A) and celery (B), high concentrations of L-AA can be seen associated with the vascular regions (large arrows) and specifically the phloem (B II). In addition, L-AA staining can be observed around the developing seeds in courgette and in the storage parenchyma in celery (small arrows).

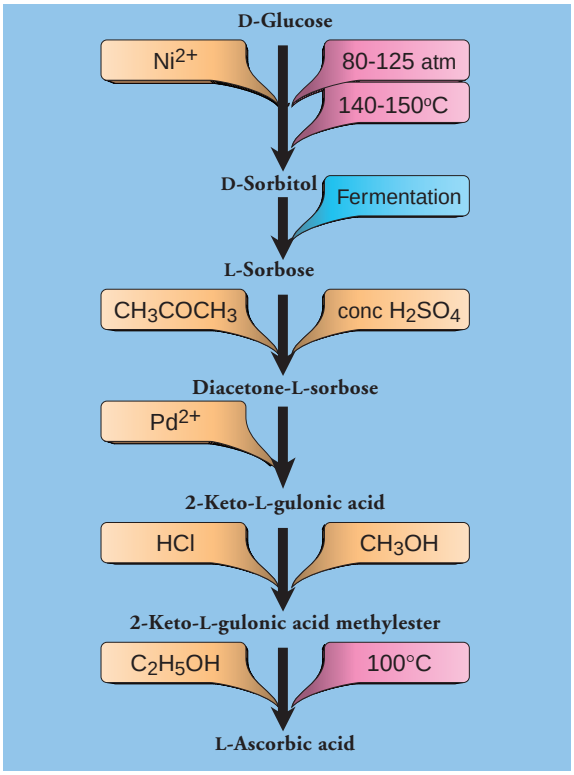

Figure 4 The Reichstein process for Bulk Manufacture of L-AA
In the widely used Reichstein process D-glucose is converted to L-AA via a series of chemical steps and a single bacterial fermentation for the conversion of D-sorbitol to L-sorbose. Catalysts for the development of alternative processes for L-AA synthesis include the use of environmentally hazardous chemicals (orange boxes) or steps requiring high energy consumption (purple boxes).

with a number of potential L-AA and D-EAA precursors (Table 4). D-EAA was present in the cells under all conditions tested and was strongly accumulated when cells were supplied with the natural precursor D-arabinose. Conversely, L-AA was only observed in cells incubated with either L-galactose, L-galactono-1,4-lactone or L-gulono-1,4-lactone (the immediate L-AA precursor in the animal biosynthetic pathway). Data obtained regarding the specificity of the enzymes involved in D-EAA biosynthesis in *S. cerevisiae* supported the hypothesis that the pathway could be 'hijacked' to produce L-AA if supplied with the appropriate precursors. This was further supported by *in vivo* labelling experiments which showed a six-fold decrease in the proportion of radioactivity incorporated into L-[^{14}C]AA from L-[1-^{14}C]galactose if the cells were preincubated with 5 g L^{-1} D-arabinose[5].

Our results demonstrate that yeast cells are capable of direct fermentation of L-galactose to L-AA. However, given that L-galactose is an extremely rare and expensive sugar a process using L-galactose as a starting material could never be economical. In order to overcome this problem, we are currently developing new yeast strains with extended metabolic competence for the synthesis of L-galactose directly from inexpensive substrates. This project is supported by the Scottish Enterprise Proof of Concept Fund and aims at developing an industrially acceptable yeast-based single-step fermentation process for the manufacture of L-AA from cheap and readily available starting materials. If successful, this development will form the basis of a new biotechnology for the manufacture of vitamin C, eventually offering a viable alternative to current production methods[6].

References

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Precursor	L-AA (mg gDW^{-1})	D-EAA (mg gDW^{-1})
D-glucose	Nd	1.67 ± 0.26
D-mannose	Nd	3.21 ± 0.38
D-galactose	Nd	0.20 ± 0.06
L-galactose	658.0 ± 94.5	0.18 ± 0.08
D-arabinose	Nd	200.8 ± 21.2
D-galactono-1,4-lactone	Nd	0.60 ± 0.14
L-galactono-1,4-lactone	865.5 ± 113.6	0.19 ± 0.04
L-gulonono-1,4-lactone	138.2 ± 10.3	0.22 ± 0.05

Cultures were grown in the dark with 5 g L^{-1} sucrose as the carbon source. Cultures were harvested and washed then resuspended in medium with the appropriate C-source (5 g L^{-1}) and incubated for a further 24 h. Samples were divided into two aliquots for the determination of cell dry weight or L-AA/D-EAA concentration by HPLC
Nd = Not detected

Table 4 Effect of Potential Precursors on Soluble Antioxidant Content of *S. cerevisiae.*