Characterisation of *Chlorella pyrenoidosa* L-ascorbic acid accumulating mutants: Identification of an enhanced biosynthetic enzyme activity and cloning of the putative gene from *Arabidopsis thaliana*

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Introduction

The major L-ascorbic acid (L-AA) biosynthetic pathway in plants was recently elucidated by Wheeler *et al.* (1998 *Nature* 393: 365) (Fig. 1) yet relatively little is known regarding the control of L-AA biosynthesis. To gain greater insight into L-AA biosynthesis in plants, we undertook a comparative study of wild-type (WT) and two L-AA accumulating strains (H1 and H2) of *Chlorella pyrenoidosa*. An enzyme catalysing a potentially rate limiting reaction was identified in *C. pyrenoidosa* and partially purified from *Arabidopsis thaliana*.

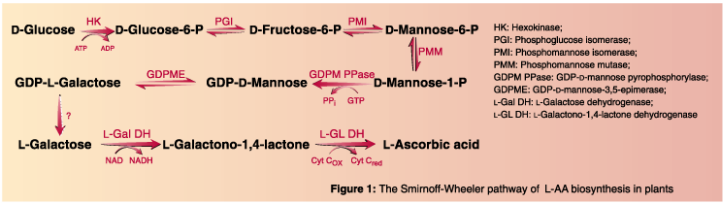


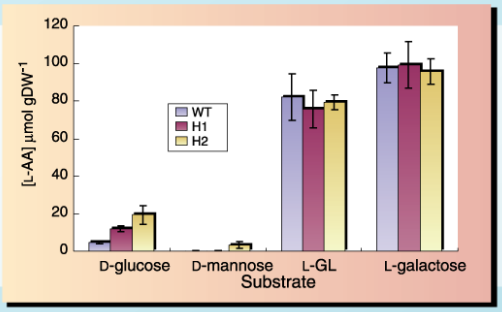
Figure 1: The Smirnoff-Wheeler pathway of L-AA biosynthesis in plants

Results

Effect of Incubation with Pathway Intermediates on L-AA Content of *C. pyrenoidosa* Strains

Cells were grown to mid-logarithmic phase then harvested, washed and resuspended with the appropriate carbon source (25 mM). Incubation was continued for 24h prior to L-AA quantification.

- Strains H1 and H2 contain enhanced L-AA levels when cultured in the presence of glucose
- D-Mannose depresses L-AA levels in all strains
- Incubation with L-galactonolactone and L-galactose results in L-AA enhancement in all three strains to similar levels
- The biosynthetic pathway in *C. pyrenoidosa* and higher plants share at least the last two steps



Incorporation of Label from ^{14}C-Intermediates into L-AA in *C. pyrenoidosa* Strains

10^{10} cells were incubated with 111 kBq of substrate in 2 ml medium for 4 h. Cells were extracted in perchloric acid and ^{14}C-L-AA quantified.

- All strains incorporate substrates L-galactose > D-mannose > D-glucose in accordance with position on the pathway
- *C. pyrenoidosa* and higher plants have a common pathway
- Both H1 and H2 incorporate more D-glucose or D-mannose than wild-type
- Incorporation of L-galactose WT = H1 > H2

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

In Vitro Activities of Smirnoff-Wheeler Pathway Enzymes in *C. pyrenoidosa* Strains

Cells were extracted in 50 mM tris pH 7.5, 5 mM DTT, 1 mM EDTA, 1 mM EGTA , 1 mM benzamidine hydrochloride and 0.5 mM PMSF. Extracts were desalted by gel filtration prior to enzyme assay.

- Wide variation in enzyme activities
- Only HK using glucose as substrate and GDP-L-gal PPPase activities were upregulated in H1 and H2
- Free L-galactose content of H1 and H2 were enhanced

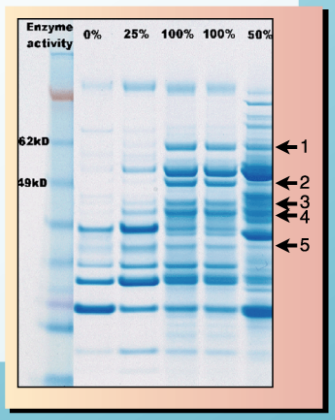
Enzyme	Specific Activity (nmol min^{-1} mg protein^{-1})[a]		
	WT	H1	H2
HK (glucose)	5.0	7.1	8.0
HK (mannose)	2.7	1.3	1.8
PGI	13.2	15.8	6.4
PMI	2.5	2.3	2.0
PMM	1.8	1.0	2.0
GDPM PPase	0.42	0.62	0.27
GDPME	0.50	2.33	0.05
GDP-L-gal Pyrophosphatase	0.04	0.38	0.61
L-Gal DH	6.1	6.9	5.9
Free [L-gal] nmol gDW^{-1}	3.62 ± 0.60	11.27 ± 5.29	11.00 ± 4.12

[a] Standard error was less than 10% in all measurements

Partial Purification and Identification of GDP-L-gal Pyrophosphatase Activity from *Arabidopsis thaliana*

GDP-L-gal PPPase activity was extracted from *A. thaliana* roots and partially purified by anion exchange and hydrophobic interaction chromatography. Fractions from the hydrophobic column were run on a 4-12% polyacrylamide gradient gel and stained with colloidal coomasie blue.

- Proteins 1-5 (with a loading pattern corresponding to enzyme activity) were sequenced by Edman degradation
- A 21 amino acid sequence from protein 2 had 100% homology to nucleotide pyrophosphatase-like protein
- The protein consists of 496 amino acids with a MW of 54.7 kDa
- The corresponding gene has been cloned from *A. thaliana*



Conclusions

- *C. pyrenoidosa* is a suitable model for L-AA biosynthesis in higher plants
- *C. pyrenoidosa* strains H1 and H2 have enhanced L-AA biosynthetic capacity
- Enhanced biosynthesis is caused by upregulation of a step between D-mannose and L-galactose
- GDP-L-Gal pyrophosphatase activity in *C. pyrenoidosa* strains H1 and H2 is enhanced when measured *in vitro*
- *A. thaliana* contains a similar enzyme activity and the corresponding gene has been cloned
- Overexpression of GDP-L-gal pyrophosphatase represents a possible target to produce plants with enhanced L-AA content

Acknowledgents
This work was funded by SEERAD.
We wish to thank Mr. Ian Pitkethly (SCRI) for help in producing this poster.
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