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*

Antonio Di Matteo1,2, Robert D. Hancock1, Heather A. Ross1, Luigi Frusciante2, Roberto Viola1

1Unit of Plant Biochemistry, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, U.K.
2Dipartimento di Scienze del Suolo della Pianta e dell'Ambiente, Università degli Studi di Napoli "Federico II", Italy

**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*.

**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
- α-Mannose depresses L-AA levels in all strains
- Incubation with L-galactono-1,4-lactone 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

![Figure 1: The Gmn pathway of L-AA biosynthesis in plants](image)

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

10^6 cells were incubated with 111 kBq of substrate in 2 ml medium for 4 h. Cells were extracted in perchloric acid and 14C-L-AA quantified.

- All strains incorporate substrates L-galactose > α-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 α-mannose than wild-type
- Incorporation of L-galactose WT > H1 > H2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Metabolised label incorporated into L-AA</th>
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<tbody>
<tr>
<td>D[14C]-glucose</td>
<td>0.06 ± 0.01, 0.27 ± 0.09, 0.57 ± 0.04</td>
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<tr>
<td>D-[1-14C]-mannose</td>
<td>2.53 ± 0.78, 10.05 ± 1.02, 1.07 ± 1.84</td>
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<tr>
<td>L-[1-14C]-galactose</td>
<td>56.68 ± 10.10, 59.92 ± 13.78, 35.82 ± 7.05</td>
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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 PPhase activities were upregulated in H1 and H2
- Free L-galactose content of H1 and H2 were enhanced

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (nmol min^-1 mg protein^-1)</th>
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<tbody>
<tr>
<td>HK (glucose)</td>
<td>5.9 H1: 7.1 H2: 8.0</td>
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<tr>
<td>HK (mannose)</td>
<td>2.7 H1: 1.3 H2: 1.8</td>
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<tr>
<td>PGl</td>
<td>13.2 H1: 15.8 H2: 6.4</td>
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<tr>
<td>PMI</td>
<td>3.3 H1: 2.3 H2: 2.0</td>
</tr>
<tr>
<td>PMM</td>
<td>1.8 H1: 1.0 H2: 2.0</td>
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<tr>
<td>GPMPPase</td>
<td>0.42 H1: 0.62 H2: 0.27</td>
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<tr>
<td>GPMME</td>
<td>0.50 H1: 2.33 H2: 0.65</td>
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<tr>
<td>GDP-L-gal Pyrophosphatase</td>
<td>0.04 H1: 0.38 H2: 0.61</td>
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<tr>
<td>L-Gal DH</td>
<td>6.1 H1: 6.9 H2: 5.9</td>
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</table>

| Free [L-gal] nmol gDW^-1 | 3.62 ± 0.60, 11.27 ± 3.29, 11.50 ± 4.12 |

*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 PPhase 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 coomassie 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*

![Image](image)

**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 α-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

**Acknowledgements**

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