Abstract

Several attempts have been made to alter the aerobic metabolism of plants, especially those related to the oxygenation or carboxylation of Rubisco. However, designing a more efficient Rubisco protein is rather problematic since its structural manipulation leads frequently to an enhancement of oxygenase activity, which is responsible for photorespiratory losses. In order to reduce oxygen availability inside the chloroplast, a chimeric gene consisting of a soybean leghemoglobin cDNA (lba) ligated to the chloroplast targeting signal sequence of the Rubisco small subunit gene, was introduced and expressed in *Nicotiana tabacum*. Lb was efficiently imported and correctly processed inside the chloroplasts of transgenic tobacco plants. Furthermore, the level of Lb expression in leaf tissue ranged from 0.01 to 0.1%. Analysis of photosynthesis, starch, sucrose and enzymes involved in aerobic metabolism, revealed that despite the high affinity of Lb for oxygen, no significant difference was observed in relation to the control plants. These results suggest that higher Lb concentrations would be required inside the chloroplasts in order to interfere on aerobic metabolism. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Leghemoglobin; Oxygen; Photorespiration; Photosynthesis

1. Introduction

In a leaf at 25°C, oxygen in the air equilibrates with dissolved O₂ in aqueous solution to produce a concentration of approximately 250 μM. Under these conditions, oxygen concentrations within photosynthesizing chloroplasts have been experimentally estimated to be 10–20% higher than the external aqueous environment, which is 275–300 μM O₂ [1]. Oxygen acts as a substrate or cofactor in many biochemical reactions in both the primary and secondary metabolism of plant cells. For example, chlorophyll and protoporphyrin biosynthesis [2], an alternative Hill oxidant: Mehler reaction [1], and substrate to the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in C₃ plants. Photorespiration in these plants is assumed to be responsible for as much as 40% loss of net CO₂ assimilated. In this manner, the photorespiration can be considered a wasteful process since the CO₂ released must be fixed again within the leaf [3].

Control of photorespiration has emerged as a primary objective in efforts to increase plant productivity in C₃ crops [4]. Elevated CO₂ concentrations generally enhance photosynthesis over the short term (days) because of higher CO₂ concen-
trations at the carboxylation site, results in an increased availability of carbohydrate for plant growth [5,6]. Nonetheless, due to practical limitations of increasing CO₂ concentrations on a large scale, many efforts have been made to modify the properties of Rubisco to reduce the oxygenation reaction, especially using molecular biology techniques. The majority of results reported so far, indicate that modification of Rubisco structure reduces the oxygenase activity as well as the carboxylase activity [7,8]. An alternative to alter the active site of Rubisco is to modify the ratio of CO₂/O₂ in the proximity of the enzyme, by targeting leghemoglobin (Lb) to the chloroplast. Lb has a very high affinity to oxygen, and is in planta synthesized exclusively in root nodules due to a symbiotic association of Rhizobium spp. with leguminous plants [9,10]. Lb functions by facilitating oxygen diffusion through the cytoplasm of bacteria-infected nodule cells to membrane-enclosed bacteroids, and thereby, protecting O₂ damage to nitrogenase [11,12].

In previous studies, transgenic tobacco plants expressing functional different hemoglobins have been produced [13–15]. It has been demonstrated that bacterial hemoglobin promoted enhanced growth and altered metabolite production in tobacco plants [14], whereas expression of functional human hemoglobin in transgenic tobacco had no phenotypic effects reported [13]. In the present work, targeting of heterologous Lb to tobacco chloroplasts was performed to determine whether Lb can be correctly imported and processed inside the organelle and to examine the effects on photosynthesis, carbohydrates, chlorophyll and the activities of enzymes involved in oxidative stress. We have shown that the presence of Lb in chloroplasts does not interfere with aerobic metabolism in transgenic plants, that may be due to the presence of insufficient quantities of Lb to alter the rate of diffusion of the high concentrations of oxygen found inside the chloroplast.

2. Materials and methods

2.1. Gene constructions

Standard procedures were used for DNA manipulations [16]. The constructs assembling the Rubisco transit peptide and Lb were made as follows.

The prbcS plasmid carries a DNA fragment corresponding to the 5’-non-coding region, and the full-length cDNA of the small subunit precursor of Rubisco from pea [17] (kindly provided by Kenton Ko, Queen’s University, Canada). Using the polymerase chain reaction specific restriction sites were added to the flanking regions of the transit peptide encoding region. Synthetic primers provided with HindIII and EcoRI sites were as follows. The rbcS1 upstream primer was 5’-CCCAAGCCTTTAAACATGGCTTCC, and the rbcS2 downstream primer was 5’-CCCCGATTCCTGCATGCATTGCAG.

After polymerase chain reaction amplification, the fragment was digested with HindIII and EcoRI and inserted into the corresponding sites of SK(+). Bluescript (Stratagene), resulting in the SK(+). rbcS plasmid.

The leghemoglobin a encoding sequence from soybean [18] (kindly provided by K. Marcher, Aarhus University, Denmark) was modified at the flanking regions by the polymerase chain reaction. Synthetic primers provided with EcoRI and BamHI cloning sites were as follows. The leg1 upstream primer was 5’-CCCCGAAATTCAAGAATATGGTTGC, and the leg2 downstream primer was 5’-CCCCGATTCCTACTAATTATG-CC.

After polymerase chain reaction amplification, the fragments were double digested respectively with EcoRI and BamHI and cloned in the corresponding sites of SK(+). rbcS (Stratagene), previously digested with EcoRI and BamHI, resulting in the SK(+). rbcS-Lb plasmid. The authenticity of this construct was verified by DNA sequencing.

To prepare the rbcS-Lb construct for tobacco transformation, the Bin2-35ScatE9’ [19] was digested with HindIII and treated with Klenow DNA polymerase so that the end was filled in. Immediately after, the plasmid was digested with BamHI to release the chloramphenicol acetyltransferase encoding sequence. In parallel, the SK(+). rbcS-Lb plasmid was digested with HindIII, filled in with Klenow DNA polymerase and further digested with BamHI, releasing the chimeric gene rbcS-Lb. This fragment was inserted into the corresponding sites of the modified Bin2-35ScatE9’ vector, producing the plant transformation vector Bin2-35SrbcS-LbE9’.

To prepare the plant transformation Bin2-35SrbcS-LbE9’ without the targeting sequence, the plas-
mid SK(+)rbcS-Lb was digested with EcoRI and BamHI, releasing the Lb gene. This fragment was then inserted into the corresponding sites of Bin2-35ScatE9 vector previously digested with EcoRI and BamHI.

2.2. Plant transformation

Transformation experiments were performed with Nicotiana tabacum as previously described [20,21]. Regenerated plants were selected for kanamycin resistance.

2.3. Plant material and growth conditions

Seeds of SRI Petite Havana N. tabacum lines were sown in a commercial substrate and fed with commercial fertilizer at least twice a week. Plants were grown in a glasshouse under a photoperiod of 14/10 h day/night and a temperature of 20/35°C. For metabolite extraction analyses, leaf discs (approximately 10 cm²) from control and four independent S₂ transformants (R3, R6, R9, and R10) were harvested from fully expanded leaves (third or fourth from the apex).

2.4. Chlorophyll assay

Leaf samples from 4-week-old plants were homogenized in 1.5 ml tubes and the chlorophyll was extracted with ice-cold 80% buffered acetone. The debris were removed and the supernatants collected. The absorbance of the chlorophyll extracts was monitored at 663 and 646 nm. The amounts of chlorophyll-a and b were calculated as described [22].

2.5. Metabolite measurements

Sucrose contents were extracted from leaf discs according to the method of [23] and determined as described previously [24]. For estimations of starch, the samples were assayed essentially as described by Jones et al. [25].

2.6. Catalase and glutathione reductase activities

Enzyme extraction was carried out as described previously by Azevedo et al. [26]. Catalase activity was determined as described by Kraus et al. [27] with some modifications [26]. Glutathione reductase activity was determined according to Smith et al. [28] with some modifications [26].

2.7. Protein quantitation

Protein concentration was determined spectrophotometrically at 595 nm as described by Bradford [29] using the Bio-Rad Protein Assay Dye Reagent with bovine serum albumin as a standard.

2.8. Fractionation of tobacco cells and protein analysis

Subcellular fractions were obtained from 10 g of leaves as described previously [19], except that homogenization was performed in 100 ml of homogenization buffer and that 0.2% (w/v) insoluble polyvinylpyrrolidone was added to the buffer.

Purification of chloroplasts and thylakoids on a continuous Percoll gradient was performed according to Bruce et al. [30].

2.9. Western blot analysis

After SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane and immunodetected with antibodies raised against purified leghemoglobin (1/1000), ribulose 1,5-biphosphate carboxylase (1/8000), followed by an anti-mice IgG alkaline phosphatase conjugate (Sigma Chemical Co).

2.10. Gas exchange measurements

Net photosynthesis, stomatal conductance, transpiration and internal CO₂ concentration were determined in fully expanded leaves of each line using a portable infrared gas analyzer (CIRAS P/P systems, Hitchin, UK). Photosynthetic active radiation (PAR) was approximately 600 μmol m⁻² s⁻¹ at leaf level. The air supply unit of the system was connected to two cylinders, one with 21% O₂ concentration and another with 1% O₂ concentration. Gas exchange parameters were first measured at ambient oxygen concentration (21% O₂). The air supply was then changed to 1% O₂ by a manual valve, and gas exchange parameters recorded once they had stabilized under non-photorespiratory conditions (approximately 2 min af-
ter the switch between the two $O_2$ concentrations. The effects of line, oxygen concentration and their interaction were analyzed by factorial analysis of variance (ANOVA) using SPSS (version 6.1.4) software.

2.11. Leghemoglobin quantitation in transgenic tobacco plants

All operations at 4°C unless stated otherwise. Leaves (1 g) of tobacco transformants (3.6, 6.6 and 9.9) were ground in 3 ml, 50 mM Tris–Cl buffer containing 2 mM PMSF and 0.2 g polyvinylpolypyrrolidone. The homogenate was filtered through miracloth and centrifuged at 14,000 g for 15 min. The clarified supernatant was assayed for protein using a dye binding protocol (BCA, Pierce Chemical Co, IL, USA) using bovine serum albumin as a standard.

Aliquots of extracts in SDS-PAGE sample buffer (Laemmli) containing 50 μg of protein and purified and quantitated leghemoglobin α were separated on a 14% denaturing gel. Separated proteins were electroblotted onto nitrocellulose membranes and probed with antibodies raised to Lba. Antigen–antibody complexes were verified by chemiluminescence, using peroxidase conjugated secondary antibodies and luminol reagent (Pierce Chemical Company, super signal) following manufacturers directions. Chemiluminescence was captured by exposing X-ray film (Kodak, BioMax) and developed on an automated developer.

3. Results

3.1. The rbcS-transit peptide targets the soybean leghemoglobin into chloroplasts

The Rubisco transit peptide (rbcS) has been used successfully to introduce foreign proteins in vivo into chloroplasts [31–33]. In the present work, a gene construct was prepared to express the soybean leghemoglobin (Lb) into tobacco chloroplasts (Fig. 1). The Rubisco transit peptide was fused to the soybean leghemoglobin α [34]. The construct (rbcS-Lb) retained the whole rbcS targeting sequence, followed by four amino acid residues of the mature protein to allow cleavage of the transit peptide that might have required the surrounding residues. As a control, Lb was prepared without the targeting sequence assuring expression in the cytosol. Both chimaeric genes were placed under the control of the 35S transcription promoter of cauliflower mosaic virus and the 3′-non-coding region of a pea Rubisco small subunit gene [35]. These genes were introduced into tobacco, using an A. tumefaciens T, plasmid-derived vector. Transgenic plants were selfed and several independent S2 plants were characterized for both constructs.

The level of Lb expression using the 35S promoter was 0.01–0.1% of total soluble proteins for rbcS-Lb plants. These values were based on the relative intensity of the immunoreactive band to authentic Lb also loaded on the gels as a standard. As can be seen in Fig. 2, there was considerable variation in the amount of Lb present in the three different tobacco transformants analyzed. Maximal amounts were seen in 6.6, which appeared to be between 0.005 and 0.5 μg of total protein, which is about 0.01% at the low end and 0.1% at the high end. The two other transformants had less than this value of Lb. Similar results were also observed with the expression of human hemoglobin in tobacco seeds driven by the 35S promoter [13].
Fig. 2. Leghemoglobin expression levels in transgenic tobacco plants. Lb quantitation was carried out as described in Section 2. Lb expression from three independent tobacco transformants (3.6, 6.6 and 9.9) was compared with purified Lb at different concentrations (0.005, 0.05 and 0.5 μg).

showed that leghemoglobin was targeted and correctly processed inside the chloroplasts (Fig. 3a), since Rubisco enrichment in the chloroplast fraction was similar (Fig. 3b). Lb enrichment in the chloroplast fraction was low, but this was to be expected since chloroplast proteins of mesophyll cells represent up to 50% of the total proteins of the leaves [35]. In plants carrying the 35S-Lb construct, Lb was found in the supernatant (cytosol) only and no Lb was detected in the chloroplast fraction. As expected, the size of the mature Lb observed for rbcS-Lb in the chloroplast fraction was larger than that of the cytosolic Lb. This difference can be accounted by the presence of four amino acid residues of mature Rubisco and one residue from the linker region.

3.2. Gas exchange

Reduced O2 concentration significantly increased net photosynthesis (P < 0.001) with increases in the range 30–35% in respect to 21% O2. There were no significant differences in net photosynthesis between lines at either O2 concentration. The Line x concentration interaction was also not significant for net photosynthesis or any other measured parameter (Table 1). Significant differences between lines and between O2 concentrations were observed for stomatal conductance and internal CO2 concentration (Table 1). Increased in stomatal conductance were approximately 8–12% at 1% O2 for all lines; the wild type and R10 were also significantly different from the other two lines at both O2 concentration. At 1% O2, internal CO2 concentration decreased (8–10%) in all lines. No significant differences were observed for transpiration in any line or between O2 concentration (Table 1).

3.3. Effect of chloroplast-lemheglobin on metabolite levels

Sucrose and starch provide confirmatory evidence over a longer time period that the photosynthetic rate is not changed. To determine the effects of the presence of soybean leghemoglobin inside the chloroplasts on carbohydrate levels, leaf samples from the control (WT) and four independent S2 F2 transformants were harvested and assayed for their sucrose (Fig. 4A) and starch (Fig. 4B) contents. The results indicate that the concentration
of sucrose and starch are not affected, suggesting that primary carbon metabolism is not altered by the presence of Lb inside the organelle. Consistent with this, was the observation that plant growth and dry weight measurements were not statistically different (data not shown). In contrast, expression of a bacterial hemoglobin in the cytosol of transgenic tobacco promoted a significant increase in plant growth and higher chlorophyll-a levels related to the untransformed plants arguing that the bacterial hemoglobin could supply oxygen for chlorophyll biosynthesis [14]. To verify whether Lb could alter chlorophyll biosynthesis, the chlorophyll-a and b concentrations in the leaves of the transgenic and control plants were analyzed. There was no modification of chlorophyll contents in any of the transformed rbcS-Lb (Fig. 4C) and 35S-Lb plants (data not shown).

3.4. Catalase and glutathione reductase activities in transgenic plants

During photorespiration, H$_2$O$_2$ is produced in the peroxisome by oxidation of glycolate and then rapidly removed by catalase (CAT, EC 1.11.1.6) inside the organelle. Previously published data has shown that there is a correlation between catalase activity and the rate of photorespiration [3,24]. Therefore catalase activity was used as an indicator of photorespiration in the tobacco plants in this study. At high light intensities, there is often an excess of PSI reduction and NADP$^+$ pools become fully reduced. Under these conditions oxygen is reduced by PSI, leading to the generation of superoxide radicals through the Mehler reaction. This superoxide radical is then rapidly disproportionated to H$_2$O$_2$ by superoxide dismutase (SOD, EC 1.15.1.1), which may be associated with the thylakoids or in the stroma. The H$_2$O$_2$ produced is quickly scavenged via the ascorbate/glutathione pathway [36]. Regeneration of these compounds is carried out by glutathione reductase (GR, EC 1.6.4.2). Therefore, CAT and GR activities have been investigated in transgenic and wild type plants. It was demonstrated that both enzyme activities were not affected by the presence of the leghemoglobin inside the chloroplasts (Fig. 5).

### 4. Discussion

Enhanced plant growth and yield by the manipulation of the photosynthetic process has been a major goal of plant physiologists. As approximately 90% of the plant dry weight is derived from CO$_2$ assimilated by photosynthesis, increasing the carboxylation reaction of Rubisco may be a great benefit to plant productivity [3]. Using molecular biology techniques, the soybean leghemoglobin a gene fused to a chloroplast targeting sequence has been introduced into the tobacco genome, in an attempt to alter the aerobic metabolism of the transgenic plants. Western blot analysis showed that the Lb was targeted to chloroplasts and the

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**Table 1**

Measurements of transpiration ($E$), stomatal conductance ($g_s$), net photosynthesis ($A$) and internal CO$_2$ concentration ($C_i$) at two concentration (21 and 1%) on the 8th leaf of each plant line

<table>
<thead>
<tr>
<th>Type</th>
<th>$O_2$ (%)</th>
<th>$A$ (µmol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>$C_i$ (µmol CO$_2$)</th>
<th>$g_s$ (mmol m$^{-2}$ s$^{-1}$)</th>
<th>$E$ (mmol H$_2$O m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>21</td>
<td>12.7 ± 0.32</td>
<td>241.8 ± 3.2</td>
<td>250.8 ± 16.4</td>
<td>3.03 ± 0.12</td>
</tr>
<tr>
<td>R3</td>
<td>21</td>
<td>12.2 ± 0.52</td>
<td>257.0 ± 2.6</td>
<td>284.4 ± 13.4</td>
<td>3.27 ± 0.13</td>
</tr>
<tr>
<td>R9</td>
<td>21</td>
<td>12.2 ± 0.47</td>
<td>252.6 ± 5.1</td>
<td>283.5 ± 20.9</td>
<td>3.24 ± 0.17</td>
</tr>
<tr>
<td>R10</td>
<td>21</td>
<td>11.5 ± 0.44</td>
<td>248.0 ± 6.2</td>
<td>249.2 ± 18.2</td>
<td>2.98 ± 0.16</td>
</tr>
<tr>
<td>Wild</td>
<td>1</td>
<td>16.5 ± 0.44</td>
<td>216.9 ± 5.3</td>
<td>270.8 ± 16.4</td>
<td>3.14 ± 0.15</td>
</tr>
<tr>
<td>R3</td>
<td>1</td>
<td>16.4 ± 0.60</td>
<td>236.9 ± 3.3</td>
<td>322.6 ± 17.9</td>
<td>3.45 ± 0.14</td>
</tr>
<tr>
<td>R9</td>
<td>1</td>
<td>16.2 ± 0.59</td>
<td>229.1 ± 6.9</td>
<td>317.8 ± 26.6</td>
<td>3.41 ± 0.20</td>
</tr>
<tr>
<td>R10</td>
<td>1</td>
<td>15.5 ± 0.59</td>
<td>225.3 ± 7.9</td>
<td>276.7 ± 23.6</td>
<td>3.13 ± 0.18</td>
</tr>
</tbody>
</table>

**ANOVA**

- Plant line: ns
- $O_2$ concentration: * $P<0.05$; ** $P<0.001$;
- Interaction: ns

* Data are means of 20 plants ± S.E. The results of analysis of variance are shown: ns indicates no difference; * $P<0.05$; ** $P<0.001$. 
Fig. 4. The metabolite concentrations in transgenic and control tobacco leaf discs. Sucrose (A) and starch (B) accumulated in leaves over the early morning (06:00 h) and the late afternoon (18:00 h) periods. Ten replicate measurements were taken from each of five different plant lines and the results are shown as the mean sucrose and starch accumulation (expressed in mmol hexose equivalents mg⁻¹ chl). Chlorophyll-α and β amounts (C) in the transgenic lines and a wild-type control. Data represents mean values from ten individual plants of each line ± S.E.

Transit peptide was correctly processed. Interestingly, in order to develop an alternative source of human hemoglobin (Hb) in transgenic plants, translocation of the Hb into chloroplasts mediated by the same Rubisco targeting sequence has been attempted. However, Hb was only observed in tobacco seeds and roots [13]. Nevertheless, in the present work it has been shown that Lb accumulated in chloroplasts, at a concentration ranging from 0.01 to 0.1% of the total extracted proteins.

It has been suggested that an efficient system of oxygen transport may alter the flux through several metabolic pathways in plant cells [12, 14, 37]. For example, during germination an increased oxygen concentration may enhance respiration and reduce the build up of toxic compounds released during fermentation [38]. On the other hand, high temperatures stimulate the oxygenation reaction of Rubisco, which is responsible for the wasteful photorespiratory process in C₃ plants. Therefore, increasing the carboxylation reaction of Rubisco by a reduction of the O₂ concentration inside the chloroplast, by the expression of Lb has been the major aim of this work. In addition, expression of Lb in the cytosol of transgenic tobacco was also attempted in order to promote higher growth, enhanced dry matter, chlorophyll content and faster germination, as observed previously by Holmberg et al. [14]. Contrary to these results, expression of Lb in the cytosol did not affect any of the parameters analyzed. This is unexpected since soybean Lb has a much higher affinity for oxygen than the bacterial hemoglobin.

It has always proved very difficult to obtain a direct measurement of photorespiration due to the reassimilation of CO₂. However, a comparison of the rates of photosynthetic CO₂ assimilation at atmospheric concentrations of O₂ (21%) and at 1–2% O₂, has been frequently used as an indirect measurement [39]. It is clear from Table 1, that there were no significant differences between the rates of photosynthetic CO₂ assimilation at the two O₂ concentrations, between the wild type and any of the three transgenic rbcS-Lb plants. Inter-
esstantly, Couture et al. [40] have demonstrated that a chloroplast Hb was located in the pyrenoid and the thylakoid region in *Chlamydomonas eugametos*. The role of the pyrenoid has been suggested as creating a microaerobic environment where the CO$_2$ to O$_2$ ratio is maintained at a high level, through the action of carbonic anhydrase, thus at the expense of the competing O$_2$ [41]. In cyanobacteria, it has been proposed that Hb is involved in delivering O$_2$ to cytochrome oxidase under microaerobic conditions [42]. What could account for the absence of any discernible effect in the transgenic rbcS-Lb plants? It may be argued that the soybean Lb is not in a functional state in order to interfere on oxygen binding kinetics or additional residues present at the N-terminus could influence the oxygen-binding activity of Lb.

First, the N-terminus in Lb is free and does not appear to influence ligand-binding properties of the protein [43]. Addition of a few to several amino acids to the N-terminus would thus not to be expected to alter the conformation of the protein, nor impact its ligand-binding properties. The residues critical to the heme pocket are on helices distant from the N-terminus. Second, the more complex tetrameric human hemoglobin was functional in tobacco chloroplasts [13], and the observation that a chloroplast hemoglobin is naturally found in the unicellular green algae [40], strongly suggest that the soybean Lb is in a functional form within the transformed plants. Similarly, the observations that functional plant Lb and Hbs have been expressed in *E. coli* [44,45], would also indicate that the chloroplast located Lb could be able to operate as an O$_2$ carrier.

Chloroplasts are organelles with a high rate of oxygen flux during the day, as oxygen is able to act as a product as well as a substrate in several metabolic reactions. To function as an oxygen carrier and to create a microaerobic environment in root nodules, the Lb exceeds the oxygen concentration by 10 000-fold [46]. It has been shown that in the transgenic rbcS-Lb plants, Lb expression varied from 0.01 to 0.1% of the total soluble protein, suggesting that to act effectively, the Lb concentration inside the chloroplast should be several times higher in order to create a gradient that removes oxygen faster than the rate of diffusion of free oxygen. Estimating a ratio of 1:30 for barley Hb when compared to myoglobin in muscle cells, Hill [47] pointed out that it would require a free oxygen concentration of less than 1 μM, before Hb became effective in oxygen diffusion. At 25°C, the O$_2$ concentration inside chloroplasts has been estimated at being between 275 and 300 μM [1]. At this concentration, extremely high levels of Lb would be required to alter the carboxylation efficiency of Rubisco and influence other oxygen-dependent metabolic pathways. This would support the observations for a lack of significant biochemical changes in the transgenic plants, despite the high affinity of the expressed leghemoglobin for oxygen.

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References


