Steady-State and Oscillating Photosynthesis by a Starchless Mutant of *Nicotiana sylvestris*

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ABSTRACT

The photosynthetic characteristics of wild type *Nicotiana sylvestris* (Speg. et Comes) were compared with those of a 'starchless' mutant NS458 that contains a defective plastid phosphoglucomutase (EC 2.1.5.1) (KR Hanson, NA McHale [1988] Plant Physiol 88: 838-844). The steady-state rate of net CO₂ assimilation (A) was studied as a function of [CO₂], [O₂], irradiance, and temperature. At 30°C with saturating light and [CO₂] and low [O₂], A for the mutant was half that for the wild type, whereas in normal air it was 90%. The irradiance and [CO₂] at low [O₂] required for saturation were lower than the values for the wild type. At 2000 microbars CO₂, 30°C, and saturating irradiance A for both the mutant and wild type was stimulated on going from 4 to 25% O₂ by at least 13%. Slow oscillations in A were readily induced with the mutant but not the wild type, provided irradiance and [CO₂] were saturating and [O₂] was low. The period, which was about 5 minutes at 30°C and decreased by about 0.67 minutes per degree, was an order of magnitude shorter than periods reported for other plants at corresponding temperatures. To achieve the full oscillation amplitude both irradiance and [CO₂] had to exceed the minimal levels for steady-state saturation. The slowness and duration of the oscillations and the metabolic simplification introduced by deleting starch synthesis makes the mutant especially suitable for investigating the regulatory processes that generate such oscillations.

The isolation of a mutant of the diploid tobacco species *Nicotiana sylvestris* grossly deficient in plastid phosphoglucomutase activity has been described (4). The kinetic properties of the defective enzyme are such that negligible amounts of starch accumulate during the day in greenhouse grown plants. Unlike the starchless mutants of *Arabidopsis thaliana* lacking in phosphoglucomutase (2) and ADP-Glu pyrophosphorylase (8) activity, the *N. sylvestris* mutant stored sufficient neutral sugars during the day to be able to grow normally under greenhouse conditions (3, 4, 6).

The major reason for isolating the mutant was to provide an experimental subject in which the complex regulatory interactions of photosynthetic metabolism have been simplified: all factors related to the partitioning of fixed C between starch formation and sucrose synthesis have been eliminated (3). In the present paper, the general photosynthetic characteristics of the mutant leaf material are compared with those of wild-type tissue. In the absence of photosynthesis at saturating [CO₂] and irradiance, exceptionally slow and prolonged oscillations in CO₂ assimilation are readily induced for the mutant, but not for the wild type.

The study of oscillations in CO₂ assimilation has a long history (10, 17, 28, 29). In 1949 Van der Veen (23, 24) showed that oscillations could be generated by exposing leaves to brief periods of darkness and studied the influence of [CO₂], [O₂], irradiance, and temperature. The appearance and disappearance of an inhibitor of photosynthesis was postulated. Subsequent theories to account for oscillations have been concerned with the competition by the two kinases of the reductive photosynthetic cycle for the same ATP pool (26), delays in ATP regeneration associated with the transthylakoid ΔpH (10, 13), and the feedback control of Pi regeneration in the sucrose synthesis pathway by Fru-2,6-P₂ (20, 22). The last explanation implies that oscillations in ATP regeneration follow changes in [Pi] amounting to one-eighteenth or less of the total Pi required by the photosynthetic process (3). Experiments involving Pi feeding, Pi sequestration, and the use of sun and shade conditions have emphasized that oscillations only occur when the Pi supply is limited (5, 18, 19, 27, 28).

In a situation where alternative explanation for oscillations are under consideration, the availability of a new experimental subject with distinctive characteristics could prove to be of great value.

MATERIALS AND METHODS

Plant Materials

Plants of wild-type *Nicotiana sylvestris* (Speg. et Comes) and mutant NS 514, isolated as 'starchless' and containing a modified plastid phosphoglucomutase (4, 15), were grown in the greenhouse in pots of a commercial mix of sphagnum moss, perlite, and vermiculite. The plants were watered daily and fertilized weekly with a complete nutrient solution. The data shown for the mutant were obtained with F₁ starchless plants from the first backcross with wild-type (female) of the original M₂ isolate, but additional experiments have been performed with starchless plants from a third backcross. Leaves of similar size were cut from mutant and wild type at about 0830 EDT from plants that had not begun to bolt. The leaves were washed with soapy water and rinsed before punching leaf discs or cutting out a leaf section.

Measurements of Photosynthesis

For steady-state measurements the standard sample was 18 leaf discs (16 mm diameter; total area, 36 cm²; about 750 mg
fresh weight) punched from three leaves. The discs were at once distributed with their lower sides up in a Petri dish and water was added to wet the discs from below (5 mL). Inverted discs were used because there are twice as many stomata on the under surface of tobacco leaves as on top (9). Photosynthetic rates measured in this way were in the same range as those measured in the ADC chamber (see below). The leaf discs were allowed to adjust to the first conditions for assay for at least 1 h before commencing measurements.

Two types of open system were employed to study steady-state photosynthesis. Where only the gas composition was varied and mutant and wild type were compared directly, a pair of 1 L fan-stirred Plexiglass chambers illuminated from above were used. The flow rate of humidified gas was about 0.5 L min\(^{-1}\) through each; relative rates were adjusted to give the same final [CO\(_2\)]. Once a steady state was established, four to six duplicate assays (requiring 20–30 min in all) were performed by noting the flow rates and sampling the entering and leaving gas streams (30). At higher [CO\(_2\)] the sample size was reduced from 5 to 1 mL to keep the measurements within the linear response range of the infrared gas analyzer and the number of assays was increased to compensate for an increase in the fractional error. To study \(\text{O}_2\) stimulations at high [CO\(_2\)] and in experiments where the temperature or the irradiance was varied, a jacketed liquid-cooled and fan-stirred Plexiglass chamber (90 mL) illuminated from below was employed (11, 30). The irradiance was adjusted with fiberglass window screens. CO\(_2\) compensation points were also determined with this chamber. When the leaf discs reached steady-state photosynthesis in the appropriate gas at 30°C and 600 \(\mu\text{mol photons m}^{-2} \text{s}^{-1}\) the ports were closed and after 4 min the chamber gas was sampled. The gas flow was restored for 8 min between determinations (\(\times 4\)).

Oscillations in CO\(_2\) assimilations were followed by using a temperature-controlled aluminum chamber (ADC, Hoddesdon, UK) that held a 10 cm\(^2\) leaf section with its upper side illuminated (12). Gas flowed over both surfaces (about 1.0 L min\(^{-1}\)) and water was supplied through the cut edges of the section.

‘Normal air’ conditions were approximated as 345 \(\mu\text{bar}\) CO\(_2\) and 22% O\(_2\). Mean values are listed \(\pm\) standard deviations unless otherwise indicated.

**RESULTS**

**Steady-State Photosynthesis**

In normal air at 30°C and 500 \(\mu\text{mol photons m}^{-2} \text{s}^{-1}\), the average photosynthetic rate for the mutant was 90% of the wild type. For example, in September the mean rates for the mutant and wild type were, respectively, 12.2 \(\pm\) 0.5 and 14.0 \(\pm\) 0.6 \(\mu\text{mol m}^{-2} \text{s}^{-1}\) (\(n = 10\) and 10; \(P < 0.0005\)). Higher values were recorded in May, lower in August, but the mutant to wild-type ratio remained the same.

The variations of photosynthesis with [CO\(_2\)] at three different [O\(_2\)] levels, 30°C, and 500 \(\mu\text{mol photons m}^{-2} \text{s}^{-1}\) is shown in Figure 1. This irradiance was saturating for the mutant and almost saturating for the wild type. The data shown were collected in two stages. A reference framework was first constructed (circled points) by 5 to 8 comparisons between photosynthesis under the conditions indicated and those in normal air. The values for the wild type in high CO\(_2\) overlapped as did those for the mutant. A two-way analyses of variance showed that the high and low O\(_2\) values for the wild type differed significantly (\(P < 0.05\)). The means for the mutant in high CO\(_2\) were not significantly different. As each comparison included a normal-air value, the results were normalized to the mean normal-air values. In the second stage, photosynthesis was measured at a series of about seven CO\(_2\) concentrations with [O\(_2\)] nominally fixed. For each curve the results from three such sequences were pooled by normalizing the data; hence, the separate sequences are not distinguished by separate symbols in the figure. Sets of four determinations of
CO₂ compensation points were also carried out with a closed system for 11, 22, and 44% O₂ at 30°C and 600 µmol photons m⁻² s⁻¹. At none of these concentrations were the slight differences between the mutant and wild-type means statistically significant; thus, the results justified extrapolating the curves in Figure 1 to coincide at zero CO₂ for a given O₂ concentration.

At high [CO₂] the assimilation rates were relatively insensitive to changes in [O₂]. This implies that photosynthesis was sink limited (14, 16), primarily by sucrose synthesis in the mutant and by sucrose plus starch synthesis in the wild type (3, 4). Under these conditions, ribulosebiphosphate carboxylase-oxygenase activity is modulated so that the rate of net CO₂ assimilation matches the sink rates (14, 16). The limiting rate for the mutant in low [O₂] was close to 60% of that for the wild type. This ratio decreased to 50% when differences in irradiance response were taken into account. At 345 µbar CO₂ the O₂ inhibition on going from 0.7 to 22% (Fig. 1, A–B) was 41% for the wild type but only 21% for the mutant. The A curves show that at 345 µbar CO₂ and low [O₂] the mutant, but not the wild type, was close to saturation. The transition to saturation for the mutant was much more abrupt than for the wild type. There appeared to be a small increase in photosynthesis with increasing [CO₂] in the region considered to be CO₂ saturated.

Although only a limited number of photosynthetic measurements were made above 1500 µbar CO₂, the general shape of the curves suggested that at 2000 µbar CO₂ there was a small stimulation of CO₂ assimilation on going from 4 to 25% O₂ for both the wild type and the mutant (curves A and B). The explanation for such effects has been the subject of recent debate (7, 17). The matter was, therefore, further investigated by a series of simple steady-state comparisons in which steady-state photosynthesis was measured alternately in 4 and 25% O₂ at 2000 µbar CO₂ and with saturating irradiance at 30°C. Table I shows the O₂ stimulation calculated from sets of three observations beginning with either 4 or 25% O₂. The stimulations were probably greater for the mutant than for the wild type. They were much larger than the small increases observed when photosynthesis was similarly compared at 1500 and 2000 µbar CO₂ at different O₂ concentrations (data not shown). Continuing the alternating sequences for two further sets of steady-state measurements (2 h) showed a decrease in stimulation with time (data not shown). Similar comparisons between photosynthesis at 25 and 47% O₂ showed a drop in photosynthesis for the wild type and a small or negligible increase for the mutant (data not shown).

The variation of photosynthesis with irradiance at 2.7% O₂, saturating [CO₂] (1520 µbar), and at 30°C was investigated (data not shown). The mutant and wild type reached saturation at about 500 and 800 µmol photons m⁻² s⁻¹, respectively. At the higher irradiance the photosynthetic rate for the mutant was slightly reduced and at low irradiances the quantum efficiency of the mutant was less than for the wild type. No such difference was observed for the A. thaliana mutant lacking phosphoglucomutase activity (18). These properties, which have been further investigated using fluorescence-quenching measurements, will be discussed elsewhere (RB Peterson, KR Hanson, unpublished data).

Figure 2 shows the variation of photosynthesis with temperature at 1.2% O₂, 608 µbar CO₂, and 850 µmol photons m⁻² s⁻¹ and also the variation in normal air at the same irradiances. For the mutant in low [O₂] the conditions were saturating for CO₂ and irradiance at all temperatures and the curve indicates the temperature dependence of limitation by sucrose synthesis. The CO₂ assimilation rate at 30°C was 3.5 times that at 15°C which is comparable to the factor of 2.2 reported for O₂ evolution by barley at saturating CO₂ and irradiance (21). (Barley is also essentially starchless [25]). The low Arrhenius activation energy of about 9.6 kcal mol⁻¹ implies that the rate is not set by a single saturated enzyme. As expected, the curves for both mutant and wild type in normal air pass through a maximum, i.e. the increase in photorespiration with temperature exceeds the increase in gross CO₂ fixation (11).

Table I. Stimulations of Photosynthesis on Increasing the O₂ Concentration from 4 to 25% O₂ at 2 mbar CO₂, Saturating Irradiance, and 30°C

<table>
<thead>
<tr>
<th>O₂</th>
<th>Mean Photosynthesis</th>
<th>Mean of First and Third</th>
<th>O₂ Stimulation</th>
<th>µmol CO₂ m⁻² s⁻¹</th>
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<tr>
<td>%</td>
<td></td>
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<tr>
<td>Mutant</td>
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<tr>
<td>Experiment 1</td>
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<tr>
<td>4</td>
<td>14.7 a</td>
<td>15.4</td>
<td>4.3 (28%)</td>
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<tr>
<td>25</td>
<td>19.7 b</td>
<td></td>
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<tr>
<td>4</td>
<td>16.1 a</td>
<td></td>
<td></td>
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<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>25</td>
<td>15.1 b</td>
<td>14.9</td>
<td>2.6 (21%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>12.4 a</td>
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</tr>
<tr>
<td>25</td>
<td>14.8 b</td>
<td></td>
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<tr>
<td>Wild-type</td>
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<tr>
<td>Experiment 1</td>
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<tr>
<td>4</td>
<td>32.9 b</td>
<td>31.5</td>
<td>5.4 (17%)</td>
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<tr>
<td>25</td>
<td>36.9 c</td>
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<td>4</td>
<td>30.1 a</td>
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<td>Experiment 2</td>
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<tr>
<td>25</td>
<td>37.9 b</td>
<td>37.3</td>
<td>4.4 (13%)</td>
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</tr>
<tr>
<td>4</td>
<td>32.9 a</td>
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<tr>
<td>25</td>
<td>36.7 b</td>
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Oscillations

Slow oscillations in CO₂ assimilation by the mutant were readily induced provided both the irradiance and [CO₂] were high and [O₂] was low. Similar results were obtained with inverted leaf discs and with leaf sections in the ADC chamber. The results shown here were obtained with the ADC chamber. It was observed early in this study (4) that the oscillation period at 30°C was about 5 min. As 30°C was within the range of normal greenhouse temperatures, and the periods were inconveniently long at lower temperatures, 30°C was employed for both steady state and oscillation studies. Figure 3 shows that the oscillations by the mutant resemble those reported for other species (19, 28) except for their duration and the length of the period. The 40 min of oscillations in panel 1 were initiated by restoring the CO₂ concentration after a period in the light with no gas flow. The initial peak at 15 s is attributable to compounds awaiting reduction or generation of ribulose bisphosphate. The brief spike before the peak is an artifact of the transit time between reference and measuring cells. Panels 2 and 3 show fluctuations initiated by restoring the light after a dark period without disrupting the gas flow. The initial peak at 1 min, attributable to compounds awaiting reduction of phosphorylation, was followed by a 4 min flat trough. The oscillations initiated at about 6 min may indicate a major discontinuity between the initial adjustments and the oscillation mode. Panels 4 and 5 show initiation by step increases in irradiance. The drift indicated by dashed lines in panels 1 and 2 was not observed when the irradiance was 735 rather than 550 μmol photons m⁻² s⁻¹.

Figure 4 shows the dependence of the oscillatory period upon temperature. The period was not influenced by the method of initiation and at a given temperature the sample variation in the period at a given temperature was not obviously correlated with sample variation in steady-state assimilation.
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Figure 4. Variation with leaf temperature of the oscillation period under near optimum conditions.

Figure 5. Variation with irradiance of the oscillation amplitudes and steady state net CO₂ assimilation rates for leaf segments of mutant leaves at 30°C in low [O₂] (0.8%) and about 410 μbar CO₂. Rates and amplitudes are expressed relative to the limiting steady-state assimilation rate of 11.6 μmol m⁻² s⁻¹. Amplitudes were calculated from peaks 2 and 3 only. The oscillation curves were recorded in the order of descending irradiance. The internal [CO₂] was about 260 μbar when the assimilation rate was 100% and increased to 325 μbars as the rate decreased. The periods decreased from 5.5 min at the higher irradiances to 3.0 min at 310 μmol photons m⁻² s⁻¹.

Figures 5 and 6 show that the saturation steady-state rates for irradiance (about 450 μmol photons m⁻² s⁻¹) and [CO₂] (about 230 μbar) were just reached, the oscillation amplitudes were only half the maximum. Maximum amplitudes were reached about 100 μmol photons m⁻² s⁻¹ higher than light saturation was reached and 60 μbar CO₂ higher than CO₂ saturation was reached.

Both of the above results can be interpreted on the assumptions (a) that when irradiance and CO₂ both saturate the rate of photosynthesis depends on the rate of Pi regeneration by the pathway of sucrose synthesis, i.e. by sink limitation (14, 16), and (b) that under oscillatory conditions the rate of Pi regeneration oscillates (20, 22). When under steady-state conditions the irradiance is reduced to the minimal amount needed for saturation, the light energy just suffices to regenerate ATP from Pi at the rate that Pi becomes available. This rate of ATP regeneration determines the rate of ribulose bisphosphate regeneration and hence the rate of CO₂ fixation, the carboxylase-oxygenase being saturated by CO₂. If Pi availability oscillates about this steady-state value, additional light energy is required for ATP regeneration to match the peak in [Pi]. When the [Pi] falls below the peak level the excess light energy is dissipated. Similarly, for the full [Pi] amplitude to be followed when excess irradiance is supplied [CO₂] must exceed the minimal concentration for steady-state saturation. It must be noted that the results do not exclude the alternative hypothesis that the oscillations arise because of a delay in the ATP regeneration process (10, 13). If this is the case, Figure 5 implies that for maximum amplitude the input energy must exceed the average energy required, i.e. there is no energy trade-off between peaks and troughs.

Whereas the higher irradiances did not reduce the amplitude, higher [CO₂] did reduce the amplitude and period (data not shown). At 580 μbar CO₂ the amplitude was not very different from that at 180 μbar CO₂ and about 10% of that at

Figure 6. Variation with temperature of the oscillation period under near optimum conditions.
mutants of both species in low $[O_2]$ were saturated at lower CO$_2$ concentrations and irradiances than the wild type (18). Sivak and Rowell have argued that these and other characteristics are associated with a reduced availability of phosphate (18). The simplification introduced by deleting starch synthesis may make it easier to determine how phosphate availability limits the photosynthetic rate and how phosphate is apportioned between metabolism and vacuolar reserves.

The finding that both the mutant and the wild type were stimulated by O$_2$ at high CO$_2$ is surprising (Table I). Sharkey and Vassey (17) showed that the analogous O$_2$ stimulation of photosynthesis by potato leaves at 22.5°C could be explained by an increase in starch synthesis with sucrose synthesis remaining essentially constant. Evidence was presented that starch synthesis increased because increased photorespiration led to a decreased concentration of 3-phosphoglycerate and thus to a reduction in the inhibition of plastid phosphoglucomutase. In the N. sylvestris mutant, therefore, there should have been no stimulation. As the plastid phosphoglucomutase is defective (4), rather than absent, some starch synthesis may occur at high [CO$_2$], but it seems unlikely that this explains the observed stimulation. If the changes in the stromal metabolites brought about by photorespiration influence the export of triose-P in exchange for Pi, the rate of sucrose synthesis could be increased (7). This assumes that sink limitation by sucrose synthesis involves both the phosphate transporter and the cytosolic pathway of sucrose synthesis and not just the latter. When starch and sucrose synthesis can occur, as in the potato leaves, starch synthesis may be favored.

The N. sylvestris mutant is unique in one important respect. The oscillations induced in the mutant (Fig. 4) are, at a given temperature, an order of magnitude slower than those induced in most other plants that have been studied. Typical published values are: barley, 2 min at 15°C, and 1 min at 20°C (22, 29); spinach, about 2.5 min at 11°C (7), 2 min at 20°C (29), and 20 s at 26°C (2); bean (Phaseolus vulgaris) 1.25 min at 23°C (14). The bean Vicia faba is unusual in having a period of 5 to 6 min at 25°C (10). The period for the A. thaliana mutant lacking phosphoglucomutase and also for the wild type was <2 min at 20°C (18). It seems unlikely that the oscillations of the N. sylvestris mutant are generated by an entirely independent metabolic mechanism as there were no indications of additional short-period oscillations at 20 or 25°C, the initiation phenomena and damping patterns were similar to those reported for other plants, the requirement that irradiance and [CO$_2$] should be saturating is common to all the examples studied, and [O$_2$], as for other plants, reduced the tendency to oscillate (19, 28).

It was noted in the introduction that different explanations have been advanced to account for oscillations in photosynthesis. Oscillation could be generated in the cytosol, the chloroplast, or by both in combination. The task of distinguishing between these alternatives is intrinsically difficult because changes generated in the one compartment must be followed by the other, e.g. if the oscillations result from the dependence of ATP regeneration on the transthyolakoid ΔpH (10, 13), both fluorescence quenching and the levels of cytosolic compounds will fluctuate; if they result from the feedback regulation of sucrose synthesis by Fru-2,6-P$_2$ (20, 22).
fluorescence quenching will fluctuate in response. The *N. sylvestris* mutant is important because the time constants for some part of the overall system differ from those for the plants usually studied; hence, the dynamic relationship between cytosol and chloroplast must be different. This topic will be discussed further when the results of fluorescence quenching studies are reported (RB Peterson, KR Hanson, unpublished data). It seems possible that in general the oscillations are determined by both the chloroplast and the cytosol and that the *N. sylvestris* mutant is a limiting case in which the cytosol is dominant. If oscillations in Pi release generated in the cytosolic pathway of sucrose synthesis are slow enough, plastid metabolism could follow Pi release in a quasi-equilibrium manner.

**ACKNOWLEDGMENTS**

I am particularly indebted to Dr. Richard B. Peterson for instruction in the use of his gas exchange apparatus, to Dr. Peterson and to Drs. Steven C. Huber and Israel Zelitch for valuable discussions, and to Carol Clark and Nancy Burns for technical assistance.

**LITERATURE CITED**

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