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Carbon Partitioning and Growth of a Starchless Mutant of *Nicotiana sylvestris*[1]

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ABSTRACT

We have further characterized the photosynthetic carbohydrate metabolism and growth of a starchless mutant (NS 458) of *Nicotiana sylvestris* that is deficient in plastid phosphoglucomutase (Hanson KR, McHale NA [1988] Plant Physiol 88: 838–844). In general, the mutant had only slightly lower rates of photosynthesis under ambient conditions than the wild type. However, accumulation of soluble sugars (primarily hexose sugars) in source leaves of the mutant compensated for only about half of the carbon stored as starch in the wild type. Therefore, the export rate was slightly higher in the mutant relative to the wild type. Starch in the wild type and soluble sugars in the mutant were used to support plant growth at night. Growth of the mutant was progressively restricted, relative to wild type, when plants were grown under shortened photoperiods. When grown under short days, leaf expansion of the mutant was greater during the day, but was restricted at night relative to wild-type leaves, which expanded primarily at night. We postulate that restricted growth of the mutant on short days is the result of several factors, including slightly lower net photosynthesis and inability to synthesize starch in both source and sink tissues for use at night. In short-term experiments, increased "sink demand" on a source leaf (by shading all other source leaves) had no immediate effect on starch accumulation during the photoperiod in the wild type or on soluble sugar acccumulation in the mutant. These results would be consistent with a transport limitation in *N. sylvestris* such that not all of the additional carbon flux into sucrose in the mutant can be exported from the leaf. Consequently, the mutant accumulates hexose sugars during the photoperiod, apparently as the result of sucrose hydrolysis within the vacuole by acid invertase.

Starch, an important end product of photosynthesis, accumulates during the day and is typically degraded at night, with the result that the supply of reduced carbon available for growth is buffered. One of the most promising approaches to elucidating the role of starch in plant metabolism is through the use of mutants that affect some aspect of starch metabolism. In *Arabidopsis*, starchless mutants that are deficient in plastid phosphoglucomutase (1) or ADP-glucose pyrophosphorylase (8) have been isolated, and, most recently, two mutants were described that are impaired in starch degradation (2). *Arabidopsis* mutants that cannot synthesize or degrade starch are functionally similar, in that starch does not serve as a diurnal storage reservoir of reduced carbon. All of the mutants exhibit growth equivalent to that of wild type under continuous light, but growth is progressively restricted (relative to wild type) as the photoperiod is reduced. Thus, starch seems to be important only during periods of darkness. The importance of starch to plant growth is also affected by nutrition, since Schulze *et al.* (10) have shown that proper starch metabolism is required for rapid growth in high nitrogen, but not in low nitrogen, when carbohydrate is in excess.

Recently, a "starchless" mutant of *Nicotiana sylvestris* was isolated that contains a defective plastid phosphoglucomutase and accumulates negligible amounts of starch (4). It behaves similarly to the starchless *Arabidopsis* mutants in that growth is progressively restricted, relative to wild type, as the photoperiod is shortened (6), and like these mutants, accumulates soluble sugars that may function as an alternative carbon reservoir in place of starch. The availability of the wild type and starchless mutant of *N. sylvestris* has allowed us to investigate further the complex interactions associated with diurnal carbon storage. Specifically, we wanted to (a) characterize the forms of carbohydrate accumulated in leaves and assess the ability of soluble sugars to compensate for the lack of starch; (b) assess the impact of different forms of carbon storage on leaf growth during the day and at night; and (c) determine the response of the wild type and mutant to altered source/sink balance.

MATERIALS AND METHODS

Plant Growth and Harvests

The wild type and starchless mutant NS458 (4) of *Nicotiana sylvestris* were grown in soil in growth chambers. The temperature in the chamber was maintained at 25°C and lights were a combination of fluorescent and incandescent lamps that provided about 350 μmol·min^{-2}·s^{-1} at plant level. The photoperiod was set at 7, 9, or 12 h as indicated in the text. In the different experiments, leaf samples (usually leaf discs obtained from either source or sink leaves) were taken at various times during the photoperiod and lyophilized prior to carbohydrate analysis.

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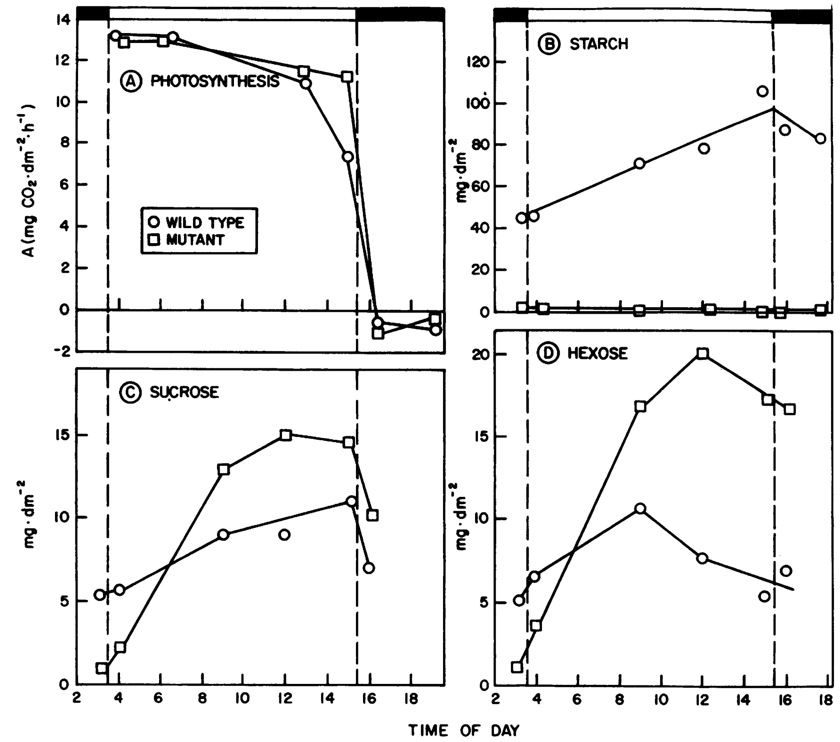

Figure 1. Diurnal changes in net photosynthetic rate (A) and accumulation of leaf starch (B), sucrose (C), and hexose (D) sugars in the wild type (O) and starchless mutant (□) of *N. sylvestris*. Plants were grown in a growth chamber under a 12-h photoperiod. Values are means of three determinations.

Assimilation Rate Measurement

Net photosynthetic rates were measured using a Li-Cor Model 6000 Portable Photosynthesis System (LiCor, Lincoln, NE).[2]

Nonstructural Carbohydrate Analysis

Lyophilized leaf discs were extracted with hot 80% ethanol. Soluble sugars (sucrose and hexose sugars) were enzymically assayed in the ethanol-soluble supernatant, and starch was measured in the ethanol-insoluble pellet after digestion with amyloglucosidase as described previously (5).

RESULTS

Carbon Partitioning in Wild-Type and Mutant *N. sylvestris*

It has been previously established that at saturating CO_2, saturating light, and low O_2, A_{max}[3] for the mutant is 50 to 65% of the wild type. These ratios are observed not only with leaf discs from greenhouse-grown plants, but also with 2- to 4-cm^2 expanding leaves from 21-d plants grown on a sucrose-containing medium in low light (K. Hanson, unpublished results). They reflect the Pi-mediated limit in CO_2 fixation set by sucrose synthesis compared with the higher limit set by sucrose plus starch biosynthesis in the wild type (3). In contrast, under normal atmospheric conditions, net photosynthesis rates are generally similar for the two genotypes (3, 6). It was also established with greenhouse-grown plants that the mutant accumulated soluble sugars in leaves during the day, whereas the wild type accumulated primarily starch (4). However, neither parameter was studied as a function of time of day, and, consequently, it is difficult to assess how the two genotypes compare in total carbon assimilation (per photoperiod) and the extent to which soluble sugars can compensate for the lack of starch.

Figure 1 compares the wild type and starchless mutant of *N. sylvestris* in terms of various photosynthetic parameters. The net photosynthetic rates (A) of attached mature leaves of the two genotypes were generally similar under atmospheric conditions (Fig. 1A). Photosynthetic rates were highest early in the photoperiod and tended to decline slightly with time of day, even though conditions were held constant in the growth chamber. The wild type had a significant level of starch in leaves at the beginning of the photoperiod, and accumulated starch at a nearly linear rate during the day (Fig. 1B). There was also some accumulation of sucrose (Fig. 1C) and hexose sugars (Fig. 1D) in leaves of the wild type during the day. In contrast, starch was essentially absent from the mutant at all times of the day (Fig. 1B). The level of soluble sugars at the beginning of the photoperiod was very low in leaves of the mutant, and there was an accumulation of both sucrose (Fig. 1C) and hexose sugars (Fig. 1D) throughout the day. In general, soluble sugar accumulation (sucrose plus hexoses) was most rapid early in the photoperiod and tended to plateau late in the day. In the experiment presented in Figure 1, accumulation of sucrose and hexose in the mutant

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[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the North Carolina Agricultural Research Service and does not imply its approval to the exclusion of other products that may also be suitable.

[3] Abbreviations: A_{max}, maximum assimilation rate; A, assimilation rate (net photosynthesis under ambient conditions).

during the day compensated for approximately one-half of the carbon accumulated as starch in the wild type.

Several other experiments were conducted in order to quantify the carbohydrates accumulated over the course of the photoperiod in leaves of the wild-type and mutant *N. sylvestris*. Measurements were made with individual mature leaves of plants grown at two different photoperiods (7 *versus* 12 h), and with leaf tissue taken from the whole canopy (12-h photoperiod only) (Table I). In general, the net photosynthetic rate of the mutant (per unit area) was similar that of the wild type, except when grown under the 7-h photoperiod. Leaves of both genotypes accumulated similar amounts of carbohydrates when grown under the 7- and 12-h photoperiods; however, in each case, total accumulation in the mutant was only about 50% of that of the wild type. In a separate experiment, carbohydrates were measured on leaf tissue taken from the whole canopy. In general, the carbohydrate accumulation was lower (relative to "mature" leaf values), reflecting the fact that both older and younger leaves were included in the sample. Nonetheless, the mutant accumulated substantially less total carbohydrate than the wild type.

Impact on Growth

Hanson and McHale (4) reported no significant difference in leaf growth between wild-type and mutant plants grown on a 16-h photoperiod; however, growth of the mutant is severely restricted (relative to wild type) when plants are grown on short photoperiods (6). The growth rate of the mutant was approximately 70 and 20% of the wild type when grown under 12- and 7-h photoperiods, respectively (6). In the present study, we found that the relative distribution of dry matter between root and shoot also differed between the two genotypes. Across three different experiments involving growth under 7-, 8-, and 9-h photoperiods, the shoot/root dry weight ratio was 2.1 ± 0.3 for the wild type and 3.7 ± 0.4 for the starchless mutant. Thus, associated with reduced overall growth was a tendency for relatively greater suppression of root growth compared to shoot growth. This contrasts with the starchless mutants of *Arabidopsis*, which are also reduced in overall growth, but have decreased shoot/root ratios relative to the wild type (10). The difference may arise from the fact that roots of wild-type *Arabidopsis* plants generally contain very little starch (10), whereas roots of *N. sylvestris* do contain significant amounts of starch (7; and S.C. Huber, unpublished data). Thus, the impact of the starchless mutation on root growth of the two species may be quite different.

Expanding leaves, like mature leaves, can accumulate starch during the day for utilization at night, so we wanted to investigate the potential impact of the mutation on leaf expansion. Plants were grown on a 9-h photoperiod and expanding leaves (approximately 30% final length) were sampled at the beginning and end of the photoperiod (Table II). In expanding leaves of both genotypes, carbohydrate levels were relatively low at the end of night as a result of utilization for growth. During the day, there was considerable accumulation of carbohydrates, with starch being the predominant carbohydrate accumulated in the wild type. The starchless mutant contained essentially only soluble sugars, and the daily accumulation of total carbohydrate was about 41% of that of the wild type. This suggested that leaf expansion of the mutant might be particularly restricted at night. Consequently, leaf expansion during the light and dark periods was monitored on 4 consecutive days.

As shown in Figure 2A, growing leaves of the wild type expanded during both the light and dark periods, but the majority of the growth occurred at night. In contrast, leaf expansion of the mutant occurred almost equally during the light and dark periods. Absolute expansion during the day was greater than that of the wild type, whereas expansion at night was reduced (Fig. 2B). Total expansion per day (light plus dark period) during the test period was 252 ± 32 mm^2 for the wild type and 195 ± 10 mm^2 for the mutant. Thus, daily leaf expansion of the mutant was about 77% compared to wild type, which corresponds very closely with genotypic difference in total leaf area (75% relative to wild type). It is possible that leaf expansion of the mutant at night is restricted

Table I. *Carbohydrate Accumulation during the Photoperiod in Mature Leaves of Wild-Type and Starchless (NS 458) N. sylvestris*

Protocol and Strain	Carbohydrate Accumulation				
	A[a]	Starch	Sucrose	Hexose	Total
		$mg \cdot dm^{-2} \cdot photoperiod^{-1}$			
Mature leaf,[b] 7-h photoperiod					
Wild type	12.7 (0.9)	49 (3)	3.2 (0.6)	5.4 (0.4)	57.6
NS 458	8.9 (0.9)	N.D.[c]	8.9 (1.4)	23.7 (0.7)	32.9
Mature leaf,[b] 12-h photoperiod					
Wild type	12.3 (0.8)	51 (7)	5.4 (1.2)	1.8 (0.6)	58.2
NS 458	13.1 (0.5)	N.D.	11.9 (3.2)	20.7 (2.4)	33.0
Whole canopy,[d] 12-h photoperiod					
Wild type		26.0	2.5	1.0	29.5
NS 458		0.5	7.9	14.2	22.6

[a] Net photosynthetic rate of attached leaves under ambient conditions expressed as mg $CO_2 \cdot dm^{-2} \cdot h^{-1}$. [b] Values are means of four to six replicates; the SE is shown parenthetically. [c] N.D. = none detected. [d] Values are means of two determinations.

Table II. *Carbohydrate Levels in Expanding Leaves of Wild-Type and Starchless (NS 458) N. sylvestris at the Beginning and End of a 9-h Photoperiod*

Values are means of these determinations.

Genotype	Carbohydrate Content							
	Start of photoperiod				End of photoperiod			
	Starch	Sucrose	Hexose	Total	Starch	Sucrose	Hexose	Total
	$mg \cdot dm^{-2}$							
Wild type	6.7	6.2	7.9	20.8	42.8	18.3	22.2	83.3
NS 458	0.1	3.6	8.2	11.9	0.3	17.1	20.3	37.7

by availability of reduced carbon reserves and that expansion during the day is enhanced because diversion of assimilates into starch does not compete with utilization for growth.

Effect of Altered Source/Sink Balance

The accumulation of soluble sugars in leaves of the starchless mutant during photosynthesis suggests that the mutant partitions relatively more carbon into sucrose (which is then hydrolyzed to hexose sugars) than can be translocated from the leaf. Thus, the system may be "sink-limited," or there may be a limitation in some aspect of the transport process (*e.g.* symplastic movement of sucrose between mesophyll cells and/or phloem loading). To distinguish between these two broad possibilities, the source/sink balance was altered by darkening three of the four largest "source" leaves on the plant. The treatment, which was intended to increase sink demand on the single illuminated source leaf, was imposed at the beginning of the photoperiod, and accumulation of carbohydrates in the test leaf was monitored during the day. The shading treatment did not affect net photosynthetic rates of the wild type or mutant (12.5 and 10.7 mg $CO_2 \cdot dm^{-2} \cdot h^{-1}$, respectively). In the wild type, starch was the predominant carbohydrate accumulated and the shade treatment did not reduce the starch accumulation (Fig. 3E). The shade treatment did reduce slightly the concentrations of hexoses (Fig. 3A) and sucrose (Fig. 3C) in wild-type leaves. However, the absolute soluble sugar pools in wild-type leaves are rather small. The decrease indicates that the treatment had some effect, but that net assimilated export was not significantly increased as a result of the imposed "demand."

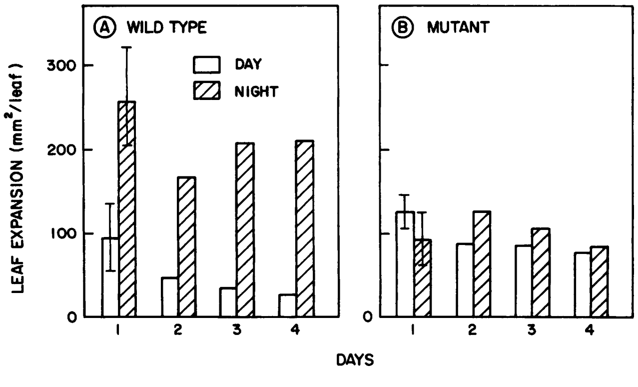

Figure 2. Expansion of rapidly growing leaves of the wild type and starchless mutant of *N. sylvestris* during the light and dark periods of 4 consecutive days. The length and width of leaves were measured at the beginning and end of the photoperiod (10 h), and area was estimated from an empirically derived standard curve relating leaf area to length. At the beginning of the experiment, leaves of the wild type and mutant were approximately 55 and 40 mm long, respectively. Values are means ± SE of four determinations.

In the starchless mutant, there was substantial accumulation of hexose sugars (Fig. 3B) and some accumulation of sucrose (Fig. 3D), and there was no effect of the shade treatment. These results are consistent with the postulate that there may be some limitation in the transport process that prevents unlimited movement of sucrose from the leaf. The lack of effect is in marked contrast to results obtained with soybean (9, 11), where starch accumulation in source leaves can respond to changes in sink demand. Alternatively, the lack of effect of increased sink demand may reflect buffering by carbohydrate reserves in other plant parts (*e.g.* stem and root), or perhaps insufficient time was given for the source to adjust to the changed situation.

DISCUSSION

We have further characterized the difference in metabolism and growth between the starchless mutant and wild type of *N. sylvestris*. By storing soluble sugars, the mutant partially compensates for the lack of starch as an end product of photosynthesis. Under normal atmospheric conditions, net photosynthetic rates per unit area of the mutant are similar to those of the wild type, and thus, the rate of carbon flux into sucrose must be increased relative to that of the wild type. Sucrose accumulates to some extent, but a significant proportion of the soluble sugars are stored as hexose sugars, presumably as a result of hydrolysis by acid invertase in the vacuole (5). The results of our preliminary source/sink manipulation experiments (Fig. 3) suggest that the capacity for sucrose movement into the phloem might be limiting. Hence, not all of the additional sucrose produced in the mutant can be exported. Both the wild type and starchless mutant of *N. sylvestris* contain high activities of soluble acid invertase in mature, sucrose-exporting leaves (5). The invertase has a relatively high affinity for sucrose (approximate K_m[sucrose] = 2.5 mM; see ref. 5) and thus would effectively hydrolyze sucrose that moves into the vacuole. Mature leaves of both the wild-type and starchless *N. sylvestris* contain similar but relatively low activities of glucose and fructose kinase (S.C.

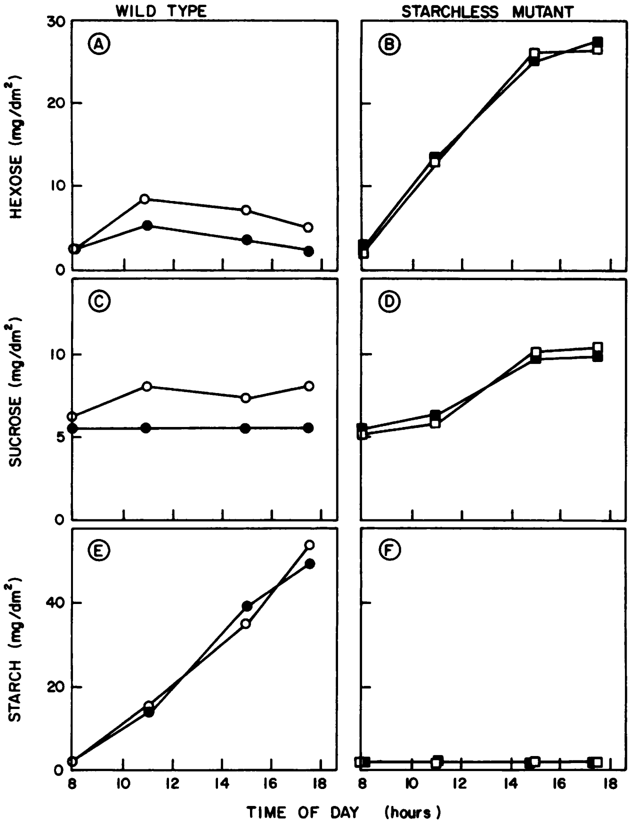

Figure 3. Effect of increased sink demand on accumulation of carbohydrates in mature leaves of the wild type (O, ●; A, C, and E) and starchless mutant (□, ■; B, D, and F) of *N. sylvestris*. All plants had about eight leaves. At the beginning of the photoperiod, three of the four largest leaves on each plant were covered with aluminum foil to increase sink demand on the illuminated leaf while the remaining four sink leaves were kept illuminated. Leaf discs were taken from the illuminated source leaf of control (O, □) or shaded (●, ■) plants at the times indicated and analyzed for carbohydrates. Values are means of three determinations.

Huber, unpublished observation). Thus, in the starchless mutant, increased cytoplasmic sucrose formation that exceeds the capacity for export from the leaf would result in increased flux of sucrose into the vacuole, where it would be hydrolyzed. The additional production of hexose sugars might easily exceed the enzymic capacity to rephosphorylate the sugars and, as a result, free hexoses would accumulate.

Because the mature wild-type and mutant leaves show very similar net photosynthetic rates during the 12-h photoperiod (Table I, Fig. 1) and the wild type accumulates more carbohydrate than the mutant for export in darkness (Table I; Figs. 1 and 3), the mutant must export more carbon than the wild type during the light period. This reversal of export priorities has a direct effect on expanding leaves. Rapidly growing leaves of the wild type receive more carbon during the dark period, and can also use their own stored carbohydrates (Table II), with the result that leaf expansion is 2.5 to 6 times greater at night than during the day (Fig. 2). The expanding leaves of the mutant receive more carbon during the photoperiod and accumulate less than those of the wild type; hence, the expansion achieved in the light and dark tend to be similar.

Growth of the starchless mutant becomes progressively restricted, relative to the wild type, as the photoperiod is reduced (6). The preceding paragraph leads to the proposal that an important aspect of the growth differential is the multiplying effect of small differences. If the previous growth history has produced mature leaves in the mutant with a total area slightly smaller than in the wild type, but with the same net photosynthetic rate per unit area (as is observed for the 10- or 12-h photoperiods), then the total carbon exported to the expanding leaves must be less in the mutant. Such a difference would also lead to less root growth in the mutant. Since the expanding leaves are partially dependent on imported carbon, they must expand less on a daily basis (Fig. 2) and are likely to be smaller at maturity than those of the wild type.

The above cycle is not in itself an explanation. It would have to be initiated by small differences in expansion in the early stages of plant growth before there are any mature leaves. This implies that starch storage and degradation in a single leaf is intrinsically more efficient than sucrose plus hexose storage. A futile recycling of hexoses and sucrose (5, 6) in the mutant would represent a hidden cost that would become more significant as the dark period increases and could be a burden to the mutant at all stages apart from the cumulative effect. There could be other hidden costs as well. When the photoperiod is reduced to 7 h, a decrease in net photosynthesis per unit area also provides an additional burden on the mutant (Table I).

Overall, these results indicate that accumulation of soluble sugars cannot completely compensate for the lack of starch in the mutant when grown on short days. The ability to accumulate a sufficient temporary reserve of reduced carbon is clearly critical for efficient plant growth, especially under short photoperiods.

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