Termination of Nutrient Import and Development of Vein Loading Capacity in Albino Tobacco Leaves

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ABSTRACT

The sink-source conversion in developing leaves of tobacco (Nicotiana tabacum L.) was studied to determine whether import termination is caused by the onset of export or is related to achievement of positive carbon balance. Albino shoots were grown in vitro and grafted to detopped stems of green tobacco plants. Termination of import was studied by providing mature leaves of the stock plant with 14CO2 and detecting the presence of labeled nutrient in developing albino leaves by whole-leaf autoradiography. In albino leaves, import terminated progressively in the basipetal direction at the same stage of development as in leaves of green shoots. Starch was not present in the plastids of mesophyll cells of mature albino leaves but starch was synthesized when discs were cut from these leaves and incubated on 3 millimolar sucrose. Import ceased progressively in developing green leaves even when photosynthesis was prevented by darkness. It was concluded that cessation of import does not require achievement of positive carbon balance and is not the direct result of export initiation.

To determine whether vein loading capacity develops in albino leaves, discs were cut from mature leaves and floated on 14C-labeled succrose solution. Uptake of label into the veins was detected by autoradiography and this uptake was sensitive to the phloem loading inhibitor p-chloromercuribenzenesulfonylic acid. However, the amount of label taken up by veins in albino leaves was less than that taken up by veins of mature green leaves.

Partitioning of photosynthetic activity is a major determinant of crop yield (7). Nutrient source-sink relationships are therefore under intensive study. The leaf is particularly interesting in this regard because it is converted, as it grows, from sink (importing) to source (exporting) status (11, 22) and comparative studies on sink-source relationships may be made within the context of a single organ.

Since dicotyledonous leaves develop in the basipetal direction, the sink-source transition occurs progressively from the tip to the base of the lamina (10, 21). Termination of import can therefore be visualized by whole-leaf autoradiography; when mature leaves are labeled with 14CO2, radioactive nutrients are transported to the base, but not the tip, of transition leaves and a distinct boundary between the two zones is apparent (21). When leaves of increasing age are autoradiographed, the progressive basipetal movement of this boundary with time is obvious. Once import has ceased, it can be induced again by darkening the leaf for prolonged periods and by manipulation of sink-source relationships (16, 19), but the amount of nutrient imported is very small in comparison to the amount transported into sink leaves and it is confined to the veins (18).

Although a considerable amount of descriptive information on the leaf sink-source transition is available, very little is known about the mechanism of conversion. The timing of the transition is closely correlated with achievement of positive carbon balance, i.e. import stops and export begins when the supply of assimilates from photosynthesis first exceeds the growth and respiratory needs of the leaf (8, 15, 23). However, it is not known whether there is a causal relationship between achievement of positive carbon balance and the timing of the transition. The results reported here demonstrate that there is no such causal relationship; although albino leaves of tobacco are incapable of photosynthesis, and therefore do not achieve positive carbon balance, they cease importing at the same developmental stage as green leaves.

MATERIALS AND METHODS

Plant Material. Seeds of the SU mutant of tobacco (Nicotiana tabacum L. cv John Williams Broadleaf) were obtained from F. W. Snyder, U.S.D.A., Beltsville, MD. Seeds were seed surface sterilized by immersion in a 0.5% (v/v) solution of NaOCl for 10 min followed by repeated washing in sterile distilled H2O. The seeds were then sown aseptically on filter paper moistened with water, and 9 d later albino shoots were transferred to agar-hardened nutrient culture medium (14) containing 1.0 mg l-1 kinetin (6-furfurylaminopurine) and 0.25 mg l-1 α-naphthalametic acid. Cultures were incubated in continuous light from fluorescent lamps (75 µmol photons m-2 sec-1) as measured by a LI 185A quantum sensor (Lambda Instruments). When the albino shoots were 1 to 2 cm in height, they were grafted to detopped green tobacco plants (cv Maryland Mammoth) and were placed in the greenhouse. Plants in the greenhouse were grown in a soil-vermiculite (1/1) mixture, and fertilized with controlled-release fertilizer (Osmocote 14/14/14, Sierra Chemical Co., Milpetas, CA). Daylength in the greenhouse was extended to 18 h with incandescent lamps (50 µmol photons m-2 s-1).

Leaf Age. Leaf age was measured using a modification of the LIPI (2). LIPI specifies the developmental age of leaves based on an arbitrary index leaf length. Under certain conditions, this index allows comparisons to be made between leaves on the same plant or between leaves of different plants (2). One of these conditions is that index leaves must be the same length at the same stage of development. Since green, light-green, and albino leaves in this study differed in size throughout development, growth curves were displaced relative to each other when LIPI was used to establish age. Therefore, a modification of the LIPI was employed to align the growth curves. The end of the exponential period of growth was chosen as a biologically meaningful reference point. Leaf area for each plant type was first plotted on semi-log paper against LIPI (2), using 20 mm as the index leaf length. The LIPI value at the point of departure of the growth curve from linearity on each semi-log plot (end of exponential

1 Abbreviation: LIPI, leaf plastochron index.
growth) was then subtracted from LPI values to yield a modified LPI. The original and modified indices, therefore, have scales of the same unit length; they differ in that the zero value is determined by leaf length in the former and by the end of the exponential growth period in the latter.

**Autoradiography of Whole Leaves.** Individual mature leaves were enclosed in polyethylene bags and exposed for 10 min, in a greenhouse, to 2 x 10^9 Bq ¹⁴CO₂ (1.9 GBq mmol⁻¹) generated from Na₂¹⁴CO₃ as previously described (21). After 4 to 6 h of transport, developing leaves were removed, frozen with powdered dry ice, and lyophilized (Virtis freeze-dryer). Dried leaves were placed in contact with Kodak XAR-5 x-ray sheets and exposed from 4 to 10 d.

**Vein Loading.** The upper and lower epidermal layers of leaves were abraded with carborundum (320 grit) and 5.6 mm diameter discs were cut, under the surface of water, with a cork borer. Discs were washed with Mes buffer (20 mM containing 20 mM CaCl₂, adjusted to pH 5.5 with KOH) for 10 min and transferred to a solution consisting of the same buffer and [U-¹⁴C]sucrose (1 mM, 9.3 x 10⁶ Bq ml⁻¹). Discs were agitated continuously under a slight vacuum on a reciprocal shaker (80 shakes min⁻¹) for 30 min in diffuse room light (9 μmol photons m⁻² s⁻¹) and washed for 30 min in five changes of the same buffer. The discs were then blotted dry, frozen in powdered dry ice, and lyophilized on a cold stage at -30°C with the condensor at -60°C. Dry discs were brought to room temperature, compressed between stainless steel plates with a clamp, and autoradiographed with Kodak AA industrex x-ray film. To quantitate [¹⁴C]sucrose uptake, dried discs were digested (13) and radioactivity was measured by scintillation counting.

**Starch Analysis.** To detect the presence of starch, leaf tissue was immersed in boiling 95% ethanol for 2 min, transferred to 95% ethanol at room temperature for 30 min, and stained with I-KI solution. Stained specimens were examined in an Ortholux II microscope.

**RESULTS**

**Import Termination in Albino and Green Leaves.** There are three phenotypes of the SU mutant of tobacco (1): heterozygous plants (Su/su) are light-green in color while homozygous plants are either green (su/su) or albino (Su/Su). When grown in soil, albino plants die as soon as food reserves are exhausted. At this stage, they are only a few mm in height. When albino shoots were grown in vitro and grafted to green tobacco plants as described in "Materials and Methods," the grafted shoots were viable for months and produced leaves continuously. In Figure 1, leaf area is plotted against leaf age as measured by a modified LPI. Under the growth conditions employed, light-green leaves

![Fig. 1. Area of green (O), light-green (●), and albino (Δ) leaves plotted against leaf age as measured by a modified leaf plastochron index. The point at which import ceases at the leaf tip and leaf base are indicated by open and closed arrows, respectively. These points are the same for the three leaf types.](image1)

![Fig. 2. Autoradiographs of developing (A) light-green and (B) albino leaves. The leaves imported ¹⁴C-labeled nutrient from mature photosynthetic leaves. The importing (black) and non-importing (white) zones are readily apparent. Scales, 1 cm. Leaves reduced in area approximately 40% by lyophilization.](image2)

![Fig. 3. Progressive termination of import in green (su/su) leaves plotted against leaf age as measured by a modified leaf plastochron index. Per cent leaf blade importing was determined by measuring, on autoradiographs, the distance from the leaf base to the import-termination boundary, dividing by total leaf length, and multiplying by 100. Scales, 1 cm. Leaves reduced in area approximately 40% by lyophilization.](image3)

...continued to grow after green leaves of comparable age were no longer expanding and, at maturity, were significantly larger than green leaves (Fig 1). Leaves of grafted albino shoots were smaller than those of green or light-green plants (Fig 1) and were, by comparison, leathery in texture. Albino leaves remained white with a slight yellow cast. Spots of Chl-containing revertant tissue, of varying size, were noted.

**Termination of nutrient import was analyzed by providing mature leaves with ¹⁴CO₂ and visualizing the import zone of developing leaves by whole-leaf autoradiography (21). In order to provide all sink leaves on the shoot with radiolabeled nutrient, it was necessary to label five mature leaves with ¹⁴CO₂ since, in...**
tobacco, phloem transport takes place preferentially along the orthostichies (9, 17). Therefore, five mature leaves, in phyllotactic sequence, were labeled with $^{14}$CO$_2$ and, 4 to 6 h later, the developing leaves were removed, frozen, lyophilized, and autoradiographed. In the case of plants with albino grafts, five mature green leaves of the stock plant were labeled with $^{14}$CO$_2$. Two autoradiographs of developing leaves are pictured in Figure 2. Distinct importing and nonimporting zones were apparent in green and light-green (Fig. 2A) leaves, as expected, and also in developing albino leaves (Fig. 2B). The developmental period during which the leaves terminated the import function is indicated by arrows in Figure 1. Import termination began at the leaf tips when the leaves were approximately 0.5% expanded and was complete, at the base of leaves, five plastochrons later at approximately 30% expansion. This means that five leaves on each plant were simultaneously losing the capacity to import, one leaf entering this phase of development as the leaf five plastochrons older was just completing it. Termination of import occurred over the same developmental time span in albino leaves as in green and light-green leaves (Fig. 1). Radioactive material was imported by mature albino leaves but it was confined to the veins as it is in importing darkened mature green leaves. This pattern is distinct from the uniform labeling of vein and mesophyll tissues which occurs in sink leaves.

At present it is not known whether the mesophyll cells of nonimporting albino leaves are completely starved for phloem-transported nutrient. Mature albino leaves were stained with I-KI solution to determine whether there is sufficient transfer of carbohydrate from the phloem to surrounding tissues to support starch synthesis. Starch grains were present in the plastids of guard cells but not in mesophyll cells. Starch was noted in mesophyll cells of revertant tissue in albino leaves. To determine whether mesophyll cell plastids of albino tissue were capable of starch synthesis, albino leaf tissue was abraded and 5.6-mm discs were floated on the surface of a 3 mM sucrose solution containing 25 $\mu$g ml$^{-1}$ ampicillin for 24 h. Starch was synthesized in mesophyll cells in all discs. If the mesophyll cells of mature albino leaves are starved, the life span of these leaves would undoubtedly be abbreviated. In fact, albino leaves became senescent and died soon after reaching maturity (LPI 6–8), whereas leaves of green plants lived much longer (>LPI 20).

Experiments were also conducted to determine whether import is terminated in leaves of green (su/su) plants in the absence of photosynthesis. Attached green leaves of age LPI $-2.4 \pm 0.2$ were totally darkened for a period of 8 d in an aluminum foil shield. The shield was constructed in two layers with fine holes on one side of the outer layer and the other side of the inner layer to allow air exchange. Radiolabeling experiments were not conducted at the beginning of the dark period but it was known from other experiments (Fig. 3) that, in leaves of age LPI $-2.4$, only the apical 10% of the leaf had stopped importing. During the 8-d dark period, the leaves aged 2.6 plastochrons and grew from an area of 15 cm$^2$ to an area of 30 cm$^2$. Undarkened control leaves grew to an area of 80 cm$^2$ (Fig. 1, LPI 0.2). At the end of the dark period, mature leaves of the plants were labeled with $^{14}$CO$_2$ as described above and the darkened leaves were autoradiographed following a 6-h import period. The boundary between importing and nonimporting zones of the leaf was approximately equidistant from the leaf tip and base in darkened leaves.

![Fig. 4](image-url)  
**Fig. 4.** Autoradiograph of a green leaf which had been darkened for 8d prior to importing $^{14}$C-labeled nutrient from mature leaves. Scale, 1 cm. Leaf reduced in area approximately 40% by lyophilization.

![Fig. 5](image-url)  
**Fig. 5.** Autoradiographs of green (A, B) and albino (C, D) leaf discs exposed to 1 mM $[^{14}]$C)sucrose for 30 min followed by a 30-min wash. Vein loading was inhibited by 2 mM PMCBS (B, D). Autoradiographs were made by exposing the x-ray sheets to leaf discs for 2 d. These autoradiographs were then used as the photographic negative; white regions therefore indicate the presence of $^{14}$C. Due to higher density in A, the photograph was exposed for half the time period of those in B to D. Scale, 1.5 mm.

| Table 1. Radioactivity in Leaf Discs after 30 Minutes of Uptake of $[^{14}]$C)Sucrose followed by a 30-Minute Wash in Buffer |
|------------------|--|---|
| Albinos          | Green |
| $0.2$ + PMCBS    | 20.8 ± 2.4 | 22.6 ± 1.4 |
| $0.2$ - PMCBS    | 49.7 ± 5.4 | 110.7 ± 5.4 |

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(Fig. 4) as it was in control leaves (Fig. 3, LPI 0.2). Therefore, import termination was not retarded by darkening.

**Phloem Loading.** The transition from the importing to the exporting function is accompanied, in green leaves, by development of phloem loading capacity (3). To determine whether albino leaves were capable of vein loading, leaf discs were cut from mature abraded leaves and immersed in [14C]sucrose solution for 30 min under slight vacuum. Label accumulated in minor veins in both green (Fig. 5A) and albino (Fig. 5C) leaf tissue though not as much accumulated in the latter as in the former (Table 1). Vein loading was inhibited in both green (Fig. 5B) and albino (Fig. 5D) tissues by the sulphhydryl-modifying compound p-chloromercuribenzenesulfonic acid (Table 1) (3).

**DISCUSSION**

In green leaves, the import-export transition coincides in time with achievement of positive carbon balance (8, 15, 23). The most obvious mechanism to account for the transition is mass action, i.e. import is terminated by the initiation of phloem transport out of the leaf. The present evidence, which demonstrates import termination in albino leaves, rules out this mechanism in tobacco. Cessation of import does not depend upon initiation of export. Of course, it is entirely possible that physiological and structural changes which cause leaf tissue to stop importing also prepare it for phloem loading and the beginning of the export phase in the event that photosynthesis is available. The results also demonstrate that import termination is not light-dependent and that green leaves will stop importing even when photosynthesis is prevented by darkening.

It is important to note that 'import termination' does not mean complete cessation of phloem transport into the leaf under all circumstances. In mature albino and darkened green leaves, imported labeled materials can be detected in autoradiographs. However, the pattern of label distribution differs qualitatively from that seen in sink leaves; radioactivity is present in the veins and is not evenly distributed throughout the lamina. Evidently, nutrients are transported into the vein network of mature leaves in response to demand but do not readily leave the veins. At present, the impediment to transport from veins to mesophyll tissues is not known. We recently tested the hypothesis that retrieval inhibits net efflux from the minor vein phloem of darkened mature green leaves (20). It was shown that sucrose exits the phloem and enters the apoplast but is then efficiently retrieved by active phloem loading. Although this mechanism partly explains the poor sink strength of mature leaves, it does not fully explain termination of import during the sink-source conversion; total efflux from minor veins in mature leaves is far below the amount needed to account for import in sink leaves (20). Another possible mechanism, as yet untested, is that symplastic transport between the phloem and surrounding tissues is blocked during the import-export transition.

It is not clear whether nutrients are completely prevented from reaching the mesophyll tissue in mature albino or darkened green leaves. If a small amount entered mesophyll cells, it could be readily respired and therefore not be detected in autoradiographs. However, if carbohydrate does reach the mesophyll from the phloem, the amount must be below that needed to sustain starch synthesis. The short life-span of albino leaves after they reach maturity may indicate that the tissue is starved. However, other unknown effects of the albino condition may also be involved. It should be noted that, in many species with variegated leaves, nonchlorophyllous regions of mature leaf blades persist for long periods of time.

Since sugar transport systems are inducible in some lower organisms (4) and transport sugars are first produced when the leaf begins to export (6, 12, 23), it was of interest to determine whether phloem loading of sucrose from the free-space is induced by export sugars in tobacco leaves. This is apparently not the case because vein loading of exogenously applied [14C]sucrose was detected in leaf discs of mature albino leaves. However, significantly less label accumulated in the minor veins of albino leaves than in those of green leaf tissue. There are a number of possible reasons for this. It may be that when minor vein phloem cells differentiate they have the capacity to accumulate sucrose at a low rate and this potential increases in response to the availability of export sugar. It is also possible that vein loading is limited in albino leaf tissue by the lack of carbohydrate which is needed as an energy source. It should also be noted that sucrose did not readily penetrate into albino leaf discs, even when the tissue was abraded. Although this problem was apparently overcome by vacuum infiltration it could, nonetheless, be responsible for the weak vein loading response.

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