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Xylem-to-Phloem Transfer of Organic Nitrogen in Young Soybean Plants[1]

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ABSTRACT

Xylem-to-phloem transfer in young vegetative soybean (*Glycine max* [L.] Merr.) plants (V4 stage) was identified as the difference in the distribution of [^{14}C]inulin, a xylem marker, and [^{14}C]aminoisobutyric acid (AIB), a synthetic amino acid, fed via the transpiration stream. Since [^{14}C]AIB was retained in the stem to some extent, whereas [^{14}C]inulin was not, the distribution of these marker compounds in each leaf was expressed as a percentage of the total [^{14}C] radioactivity recovered in the foliage. The developing third trifoliolate was a consistent and reliable indicator of xylem-to-phloem transfer. The phloem stream provided to the developing trifoliolate up to fourfold the relative proportion of solute received from the xylem stream; this was markedly reduced by increased light intensity and consequently water flow through the xylem. Evidence from heat girdling experiments is discussed with respect to the vascular anatomy of the soybean plant, and interpreted to suggest that direct xylem-to-phloem transfer in the stem, in the region of the second node, accounted for about one-half of the AIB supplied to the developing trifoliolate, with the remainder being provided from the second trifoliolate. Since AIB is not metabolized it seems likely that rapid transfer within the second trifoliolate occurred as direct veinal transfer rather than indirect cycling through the mesophyll. This study confirmed that xylem-to-phloem transfer plays a major role in the partitioning of nitrogen for early leaf development.

Evidence from modeling studies indicate that the major transfer process involved in providing nitrogen to young developing organs is xylem-to-phloem. Xylem-to-phloem transfer was suggested from the enrichment of amino compounds relative to sugar in phloem sap collected from terminal stem tissues, inflorescences or fruit stalks compared to that from petioles (9, 13). This process could involve direct transfer of xylem-borne products in an unmetabolized form in the stem or in the leaf veins or indirect transfer after transformation and possibly storage in the leaf mesophyll (13).

Recently, van Bel (1) determined xylem-to-phloem transfer in tomato plants as the difference between [^{14}C]inulin and [^{14}C]AIB[2] recovery in a particular leaf. Since inulin uptake by the stem was low and could be flushed from the stem by transpirational washings with water, it was thought to be a reliable indicator of xylem transport. Based upon the linear uptake with time of inulin by leaves, amino acid redistribution during the experimental period was apparently unimportant, though it is doubtful whether the method would adequately detect redistribution of a small magnitude. AIB, which was not metabolized, was retained to some extent by the stem, leading van Bel (1) to postulate that AIB was directly transferred from xylem to phloem in the stem, and moved into young leaves.

In this report we described the applicability of van Bel's technique to the soybean plant. Young vegetative plants provided a simple system for demonstrating xylem-to-phloem transfer. Heat-girdling of the stem or petioles was used to identify sites of direct and possibly indirect transfer, while varying light intensity allowed the manipulation of the relative phloem supply to a developing indicator trifoliolate.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* [L.] Merr. cv Maple Arrow) plants, effectively nodulated with *Bradyrhizobium japonicum* (the Nitragin Co., Milwaukee, WI), were grown in medium-textured vermiculite. Plants were grown in a naturally lit greenhouse in the summer, while during the winter the daylight was supplemented with high pressure sodium lamps (120 μmol m^{-2} s^{-1} at pot level) to give a 16 h extended day and an 8 h night. The plants were watered twice weekly with one-quarter strength Hoagland-type nutrient solution minus nitrogen. Plants at the V4 stage (6) were used for all experiments. At this stage the plant has two unifoliolates, one mature trifoliolate, one almost fully expanded trifoliolate, one immature trifoliolate leaf about one-third developed, and a vegetative bud at the apex.

Plant Preparation

Soybean shoots were cut under water just above the root region. Prior to each experiment detached plants were kept undisturbed in deionized water in a controlled environment chamber for at least 1 h before transfer to ^{14}C-labeled xylem sap. The controlled environment chamber was maintained at 25°C, 40% RH and PPFD of 1200 μmol m^{-2} s^{-1} at the level of the unifoliolates.

Feeding of ^{14}C Compounds

Xylem sap (pH 6.0), previously collected and stored frozen, was diluted 10-fold with distilled water and spiked (18.5 kBq/

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[2] Abbreviation: AIB, α-aminoisobutyric acid.

Table I. *Distribution of Total ^{14}C Recovered in Vegetative Soybean Shoots Fed via Transpiration Stream with [^{14}C]Inulin or [^{14}C]AIB for 0.5 h followed by a Chase Period in Unlabeled Xylem Sap (0–2 h)*

Data represent the mean ± SE (n = 6).

Compound	Plant Part	Chase Period (h)			
		0.0	0.5	1.0	2.0
		% total ^{14}C recovered			
[^{14}C]Inulin	Stem	4.2 ± 0.3	3.3 ± 0.9	2.3 ± 0.4	2.8 ± 0.6
	Petioles	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.1
	Leaves	95.5 ± 0.3	96.4 ± 1.0	97.2 ± 0.5	97.0 ± 0.6
[^{14}C]AIB	Stem	32.7 ± 1.1	24.7 ± 2.2	27.7 ± 2.1	20.9 ± 1.6
	Petioles	7.5 ± 0.5	7.2 ± 1.0	6.2 ± 0.2	5.9 ± 0.3
	Leaves	59.9 ± 1.1	68.1 ± 3.1	66.1 ± 2.3	73.2 ± 1.5

mL) with [carboxyl-^{14}C]inulin or α-[1-^{14}C]AIB (purchased from New England Nuclear, 96 MBq/g and 1850 MBq/mmol, respectively). These solutions were fed to cut transpiring shoots in the controlled environment chamber under a variety of time course conditions. Initially, plants were fed continuously from 0.5 to 2 h or fed for 0.5 h followed by a chase period up to 2 h in unlabeled diluted xylem sap. Upon completion of these experiments a standard labeling time of a 0.5 h feed followed by a chase of 2 h was chosen for heat-girdling studies. The distribution of ^{14}C from inulin and AIB was compared in control plants and plants that were girdled (5) on the stem immediately above one of the nodes or on the petioles of the unifoliolates or of one of the trifoliolates (3 mm away from the stem). In another set of experiments the shoots were fed for 0.5 h followed by a chase of up to 2 h in unlabeled diluted xylem sap under two light regimes, a high (2150 μmol m^{-2} s^{-1}) and a low (310 μmol m^{-2} s^{-1}) PPFD. Each experimental set was composed of four separate and complete experiments, with a total of eight plants for each variable (*e.g.* time, inulin *versus* AIB).

Assay of ^{14}C in Plant Parts

Following the feed and chase, each plant was immediately divided into the stem, petioles (including petiolules) and individual leaves. To precipitate proteins and pigments associated with protein each plant part was ground in 5 volumes of cold sulphosalicyclic acid (30 mg mL^{-1}) using silica sand and a chilled mortar and pestle. The homogenate was placed in 1.5 mL micro test tubes and spun at 17000g in an IEC Centra Microcentrifuge (model 2399) for 10 min. The supernatant was retained and the pH adjusted to 6.5 with 0.1 N NaOH, removing any color that might interfere with the ^{14}C determinations by liquid scintillation spectrometry. To a 100 μL aliquot of the supernatant 0.4 mL of water and 3 mL of Beckman Ready-Solv HP/b scintillation fluid was added. The absence of color quenching was confirmed by the addition of a known amount of ^{14}C-radioactivity to plant extracts. The pellet from the tissue homogenate was retained and the ^{14}C-content determined in the protein fraction after solubilization of the pellet with 0.1 N NaOH (12); less than 6% of the total ^{14}C recovered was found in this fraction. This method, which permits the extracts to be rapidly analyzed for amino acids by HPLC (12) in follow up studies, resulted in 96 to 98% recovery of the ^{14}C from inulin or AIB taken up in the transpiration stream.

Statistical Treatment

Data were analyzed in a two-way ANOVA (Statistical Analysis System, SAS Institute, Cary, NC) to determine if there was any day effect or interactions between the different variables. No day effect was found and therefore the data from four experiments (n = 6–8 plants) were pooled to determine the mean ± SE. Treatment of the data was conducted using the Student's t-test with confidence levels set at 95%.

RESULTS

The soybean plants used in this study had a mean fresh weight in milligrams (±SE) of 739±10 for the stem, 257±5 for the petioles and petiolules, and 1318±14 for the leaves. Plants that wilted or did take up artificial xylem sap were discarded and not included in the analyses.

Regardless of whether [^{14}C]inulin was fed continuously (data not shown) or as a feed and chase (Table I) via the transpiration stream, relatively little ^{14}C was recovered outside the leaves. The proportion of [^{14}C]inulin recovered in the stem after feeding for 0.5 h was decreased linearly up to 50% with a chase period of 1 h in unlabeled xylem sap (Table I). In contrast, 27 to 50% of the ^{14}C from AIB was found outside the leaves (Table I, data not shown). Since the degree of retention by the stem of the two ^{14}C-compounds differed, a comparison of their distribution in the leaves was simplified by expressing the ^{14}C-content of each leaf as a percent of the total ^{14}C recovered in the leaves (Fig. 1). Using the feed/chase technique there was no difference between the two solutes in the percentages found in the unifoliolates and in the bud. However, after 2 h the first trifoliolate contained more [^{14}C]inulin than [^{14}C]AIB, and the second and third trifoliolates contained more [^{14}C]AIB than [^{14}C]inulin. Similar trends were found in plants supplied continuously with the ^{14}C-compounds (data not shown). However, because it is relatively inexpensive, the feed/chase technique was chosen as the standard methodology for the remaining experiments.

In contrast to the studies above, in the remaining studies control plants did not show significant differences in the

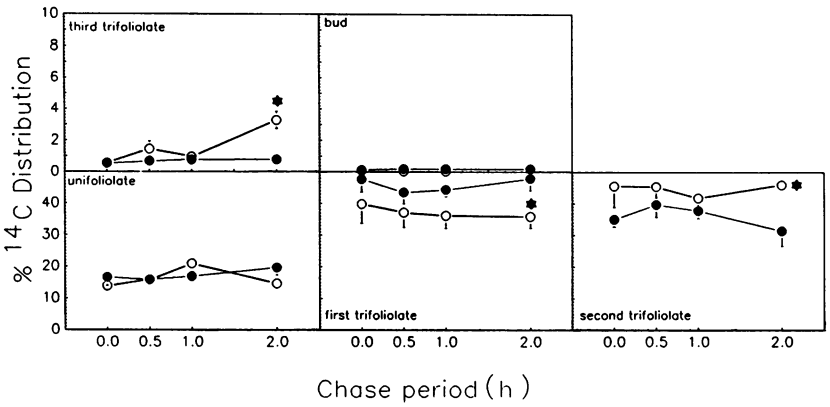

Figure 1. Distribution of ^{14}C in leaves of soybean shoots fed via the transpiration stream for 0.5 h with [^{14}C]inulin (solid symbols) or [^{14}C]AIB (open symbols) followed by a chase period (up to 2 h) in unlabeled xylem sap. Data are the mean ± SE. Where the SE bar is not shown it is within the symbol. (*), Significant difference between the two solutes.

distribution of [^{14}C]inulin and [^{14}C]AIB in the first and second trifoliolates (data not shown). However, the difference was always evident in the third trifoliolate (Fig. 2).

Girdling the stem just above particular nodes had no effect on the degree of retention of [^{14}C]inulin and [^{14}C]AIB in the stem (data not shown). Girdling also had no effect on the distribution of [^{14}C]inulin between the leaves (data not shown). It did, however, change the distribution of [^{14}C]AIB between the leaves. In particular, girdling above the third and second nodes reduced the percentage of ^{14}C in the third trifoliolate from AIB to that seen for inulin (Fig. 2). Although stem girdling above the first node did not significantly reduce the percentage of [^{14}C]AIB recovered in the third trifoliolate, it did reduce the difference between the percentage of [^{14}C] AIB and [^{14}C]inulin distribution by about 50% compared to the control treatment.

Similarly, girdling the petioles of each leaf also did not significantly affect the relative distribution of [^{14}C]inulin or [^{14}C]AIB between the stem, petioles, and leaves, or the distribution of [^{14}C]inulin between the leaves (data not shown). By contrast, girdling the petiole of the second trifoliolate, located

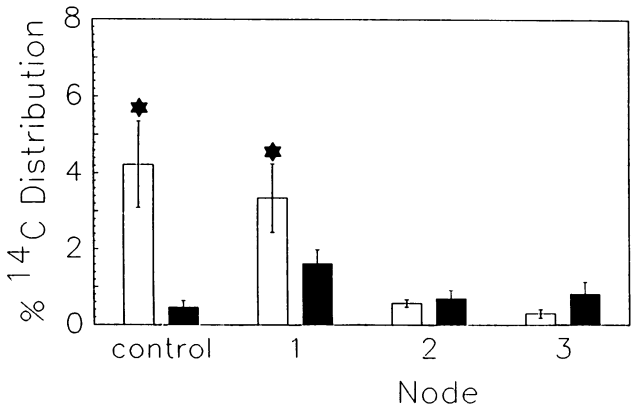

Figure 2. Influence of stem girdling on the proportion of total leaf ^{14}C recovered in the third trifoliolate of soybean shoots fed via the transpiration stream for 0.5 h with [^{14}C]inulin (solid bars) or [^{14}C]AIB (open bars) followed by a chase period for 2 h in unlabeled xylem sap. Control plants were not girdled whereas in experimental plants the stems were heat-girdled above the first (1), second (2), or third (3) nodes. Data are the mean ± SE. (*), Significant difference between the two solutes.

at the third node, reduced the difference between [^{14}C]AIB and [^{14}C]inulin in the third trifoliolate by about 60% (Fig. 3). Girdling the third trifoliolate petiole reduced the percentage of [^{14}C]AIB in the third trifoliolate to the level found for [^{14}C] inulin.

Studies in which the plants were exposed to two light regimes during the feed/chase periods indicated that the higher PPFD (2150 *versus* 310 μmol m^{-2} s^{-1}) increased the rate of transpiration by about 80% (data not shown). In both cases [^{14}C]AIB recovery in the third trifoliolate was greater than that of [^{14}C]inulin after 2 h (Table II). However, the lower PPFD resulted in a higher percentage of [^{14}C]AIB recovery in the third trifoliolate.

DISCUSSION

This study indicated that the technique described by van Bel (1) for measuring xylem-to-phloem transfer in tomato plants, can also be used in soybean plants. Evidence of transpirational flushing with unlabeled xylem sap, of low stem retention of [^{14}C]inulin, and of no influence of stem or petiole girdling (Figs. 2 and 3) on the distriubtion of [^{14}C]inulin suggest that the movement of inulin in soybean (Table I), as in tomato, was restricted to the xylem pathway. No evidence has been found for the production of $^{14}CO_2$ from soybean tissues incubated with [^{14}C]AIB [4], confirming the previous finding for tomato that AIB metabolism is insignificant (1). Thus, the difference in the distribution of ^{14}C from inulin and AIB should provide an indication of xylem-to-phloem transfer in soybean.

In lupin, amino acids that undergo xylem-to-phloem transfer in an unmetabolized form are thought to do so exclusively in the stem and in leaf veins (direct transfer), thereby bypassing the leaf mesophyll (11, 13). The proportion of these amino acids that cycles through the mesophyll is metabolized, resulting in the redistribution in phloem of excess C and N in other forms (indirect transfer). Since AIB is a synthetic amino acid and is not metabolized, it is possible that the difference in the distribution of [^{14}C]inulin and [^{14}C]AIB reflects direct xylem-to-phloem transfer only. As a precaution, van Bel (1) suggested, from the linear accumulation up to 3 h, of ^{14}C in the leaves from [^{14}C]valine uptake, that redistribution was of no importance during the 2 h experimental period. However, previous research has shown that valine predominantly undergoes direct and not indirect transfer (11, 13), and thus may

not be a reliable indicator of redistribution. Here, we preferred to use a girdling technique to differentiate between direct stem transfer, and direct (vein) and indirect (mesophyll) transfer within the leaf.

The retention of [^{14}C]AIB by the stems of tomato (1) and soybean (Table I) suggests that the stem is involved in xylem-to-phloem transfer. However, since a larger portion of the [^{14}C]AIB is retarded in comparison to [^{14}C]inulin, an underestimate of xylem-to-phloem transfer can occur if the data are expressed on a specific activity basis, as was done with tomato plants in van Bel's paper (1). This problem would be more evident in soybean, where the stem and petiole retention of [^{14}C]AIB was considerably higher than that in tomato (1). Consequently, we have compared the distribution of the two ^{14}C-labeled compounds by expressing the ^{14}C-content of each leaf as a percent of the total ^{14}C recovered in the foliage.

The distribution of the two ^{14}C-labeled marker compounds did not discriminate clearly the functional role of the first and second trifoliolates. In the initial studies (Fig. 1) the [^{14}C] inulin recovered in the first trifoliolate was about 40% greater than [^{14}C]AIB, suggesting that this leaf was not a net importing

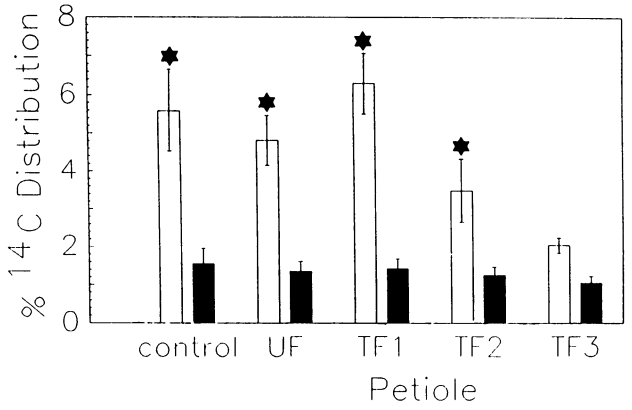

Figure 3. Influence of petiole girdling on the proportion of total leaf ^{14}C recovered in the third trifoliolate of vegetative soybean shoots fed via the transpiration stream with [^{14}C]inulin (solid bars) or [^{14}C] AIB (open bars) for 0.5 h followed by a chase period of 2 h in unlabeled xylem sap. Control plants were not girdled; in experimental plants the petioles of the unifoliolates and of the first, second, and third trifoliolates are depicted as UF, TF1, TF2, and TF3, respectively. Data are the mean ± SE. (*), Significant difference between the two solutes.

Table II. *Influence of Photosynthetic Photon Flux Density on Proportion of Total ^{14}C-Radioactivity Recovered in Third Trifoliolate of Vegetative Soybean Shoots Fed via Transpiration Stream with [^{14}C]Inulin or [^{14}C]AIB for 0.5 h followed by a 2 h Chase in Unlabeled Xylem Sap*

The data represent the mean ± SE. Numbers not sharing the same letters are significantly different at the 95% level of confidence.

Compound	PPFD	Third Trifoliolate
	$\mu mol\ m^{-2}\ s^{-1}$	% ^{14}C recovered in leaves
[^{14}C]Inulin	2150	1.4 ± 0.1^a
	310	2.0 ± 0.3^a
[^{14}C]AIB	2150	3.4 ± 0.6^b
	310	5.0 ± 0.7^c

leaf. The second trifoliolate, which contained about a third more [^{14}C]AIB than [^{14}C]inulin, appeared likely to be a net importing leaf. However, in the remaining studies, plants exposed to an identical feed and chase period (control plants) did not exhibit differential labeling of the first and second trifoliolates by [^{14}C]inulin and [^{14}C]AIB (see "Results"). Previously, van Bel (1) found no difference between the specific activity of [^{14}C]inulin and [^{14}C]AIB recovered in the basal leaves of tomato plants. As described in "Materials and Methods," the soybean plants used here were grown in the greenhouse under natural conditions of photoperiod and light intensity, except during the winter months. It appears, as suggested earlier (14), that such changing environmental conditions can influence translocation processes, even though the plants were chosen from a similar growth stage and did not exhibit much variation in fresh weight of the various parts.

In contrast to the first and second trifoliolates, the third trifoliolate always had a significantly greater portion of total leaf [^{14}C]AIB than [^{14}C]inulin (Figs. 1–3; Table II) indicating that it was a net importing leaf. Thus, this leaf was a consistent and reliable indicator of xylem-to-phloem transfer in V4 soybean plants. Since recently disturbed plants (up to a maximum of 175 min) are known to exhibit depressed phloem transport velocities (7), it is possible that the magnitude of xylem-to-phloem transfer reported in this study is an underestimate. Xylem-to-phloem transfer of [^{14}C]AIB to the indicator trifoliolate was decreased by about one-third by doubling the transpiration rate (Table II). Similar observations with tomato (1) led van Bel to suggest that xylem-to-phloem transfer is reduced because increasing transpiration rates decrease lateral escape of amino acids to xylem parenchyma cells.

Stem girdling above node two resulted in the complete inhibition of xylem-to-phloem transfer (Fig. 2), suggesting that the second node and/or the first internode represent the major location of transfer. This proposal is supported by anatomical evidence showing that the three vascular bundles of the trifoliolates extend downward through two or more internodes before anastomosing with the lateral traces of other leaves (2, 3). Xylem-to-phloem transfer in the stem, probably involving transfer cells at the departing leaf trace (1) or in the internodal region (8), could reduce the [^{14}C]AIB received in the first trifoliolate (located at the second node), in comparison to [^{14}C]inulin (Fig. 1).

Girdling the petiole of the second trifoliolate reduced xylem-to-phloem transfer to the developing trifoliolate by about one half (Fig. 3). Evidence discussed above and that from Thrower (15), indicates that this leaf, which is almost fully expanded, does not extensively import via the phloem. Therefore, it was likely that the petiole girdles primarily affected export. Since the leaf vascular bundles merge with the main stem bundles below the insertion of the primary leaves on the stem (2, 3) the phloem solution must move down the stem before moving upward to the third trifoliolate. Thus, solutes delivered in the phloem to the third trifoliolate after either direct veinal transfer or indirect transfer through the mesophyll within the second trifoliolate would be sensitive to stem girdling above all nodes. Considering the discussion above, this process would be inhibited by girdling above the first node without complication from direct stem xylem-to-phloem

transfer. A similar degree of inhibition (about 50%) of xylem-to-phloem transfer to the developing trifoliolate by girdling the stem above the first node (Fig. 2) or the petiole of the second trifoliolate (Fig. 3) supports this view.

Caution in the strict interpretation of results from petiole girdling is necessary because of potential for feedback inhibition of the translocation and photosynthetic capacities of other source leaves. However, in this study, girdling the petioles of the primary leaves and the first trifoliolate did not significantly affect the [^{14}C]AIB distribution between the leaves (data not shown) or the xylem-to-phloem contribution to the developing trifoliolate (Fig. 3). Indeed, Mayoral *et al.* (10) have shown in cucumber plants that the effect of short-term petiole girdling (less than 6 h) on translocation and photosynthetic processes is confined to the treated leaf. The present data, therefore, are interpreted to suggest that transfer within the second trifoliolate and transfer within the stem each account for approximately one-half of the total xylem-to-phloem contribution to the third trifoliolate.

It is concluded that in the V4 soybean system described here, xylem-to-phloem transfer plays a major role in the partitioning of nitrogen for early leaf development. The evidence suggests that rapid transfer at two locations is involved: direct transfer in the stem, and direct or indirect transfer within the second trifoliolate. Because AIB is not extensively metabolized, the leaf transfer process may be intepreted as direct veinal transfer. We recognize the possibility that in an intact soybean plant, the phloem stream leading from the second trifoliolate may provide solutes downward to the root system rather than upward to the developing trifoliolate. This, however, does not minimize the need or the potential for transfer of xylem-borne nitrogenous compounds to the phloem.

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