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Short Communication

An *In Vivo* Technique for the Study of Phloem Unloading in Seed Coats of Developing Soybean Seeds

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ABSTRACT

A technique has been developed which permits mechanistic studies of phloem unloading in developing seeds of soybean (*Glycine max* cv Clark) and other legumes. An opening is cut in the pod wall and the embryo surgically removed from the seedcoat without diminishing the capacity of that tissue for assimilate import, phloem unloading, or efflux. The sites of phloem unloading were accessible via the seedcoat apoplast and were challenged with inhibitors, solutes, buffers, etc., to characterize the unloading process.

Unloading is stimulated by divalent metal chelators and diethylstilbestrol, and inhibited by metabolic uncouplers and sulfhydryl group modifiers. Solutes released from the seed coat had a carbon/nitrogen ratio of 31 milligrams carbon per milligram nitrogen; sucrose represented 90% of the carbon present and various nitrogenous solutes contributed the remaining 10%. Unloading could be maintained for up to 8 hours at rates of 0.5 to 1.0 micromoles per hour, providing a valid, convenient *in vivo* technique for studies of phloem unloading and seed growth mechanisms.

The study of solute exit from the phloem ('unloading') in sink tissue has been severely hampered by the lack of convenient, valid techniques. Access to the phloem for experimental manipulation of the unloading process has been gained in but few systems, most notably sugar cane stalks (6) and *Cuscuta*-parasitized stems of *Vicia faba* (16). While much has been learned of the kinetics of photosynthate import, anatomy of the tissues involved, and uptake of assimilates by sink consumer cells (4, 5, 15), little is known of the mechanisms controlling the exit of solutes from the phloem sieve tubes.

The phloem retains its solutes with minimal radial exchange; sites of unloading are notable exceptions. A popular hypothesis is that the sieve tube/companion cell/phloem parenchyma complex counters leakage by continuously reloading assimilates along the entire vascular length, and that sinks permit unloading by locally inhibiting the reloading mechanism (5, 10). This model is consistent with the data suggesting passive efflux from the phloem of developmentally immature sinks incapable of reloading, and for storage sinks where rapid hydrolysis or compartmentation prevents reloading of sucrose (4, 5, 15). However, other studies have provided evidence that, under some conditions, sinks directly effect solute exit from the phloem complex in a controlled, energy-dependent manner (13, 16). Given the diversity of sink tissues which exist, it is probable that more than one unloading mechanism operates.

This paper characterizes a novel technique for studying phloem unloading and maternal/embryo transfer of photosynthate in legume fruit.

MATERIALS AND METHODS

Soybean (*Glycine max* cv Clark) were grown as previously described (14) except that some plants were grown symbiotically by inoculating the seed at sowing with an appropriate strain of *Rhizobium japonicum* and deleting nitrogen from the nutrient solution. Plants were utilized for experiments at various stages of seed development. The formation of a funicular abscission layer in nearly mature seeds adds considerable difficulty to the technique. Best results are obtained with seeds of 100 to 300 mg fresh weight, but with experience, the technique can be used on seeds of almost all ages.

Routine surgical operations were as shown in Figure 1 and as follows: a 2-cm incision was made along (but not touching) the pod dorsal bundle. Midway between the dorsal and ventral bundles (12), another incision was made parallel to the first. The cuts were joined and the pod wall flap removed to expose the distal halves of the attached seeds. These were carefully cut parallel to the pod wall incision and the excised distal seed halves removed. Half of each seed remained attached to the pod vascular bundles via the funiculus. The remainder of the embryo was removed by inserting a 2-mm-wide spatula between the cotyledons and lifting with a gentle twisting motion. A drop of water on the site facili-

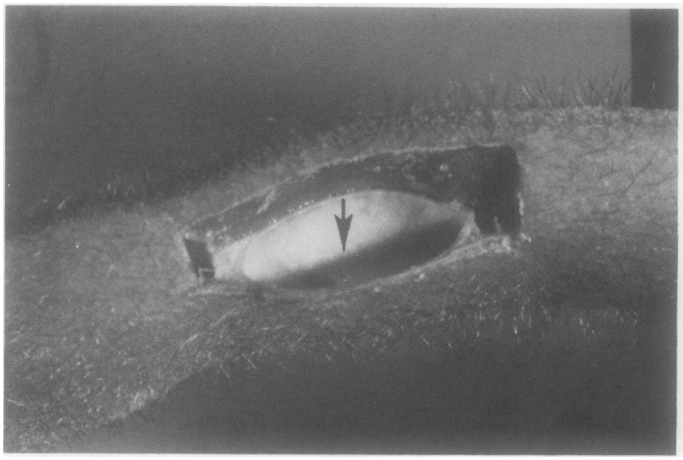

FIG. 1. Photograph of the experimental system. The arrow indicates the seed coat cup.

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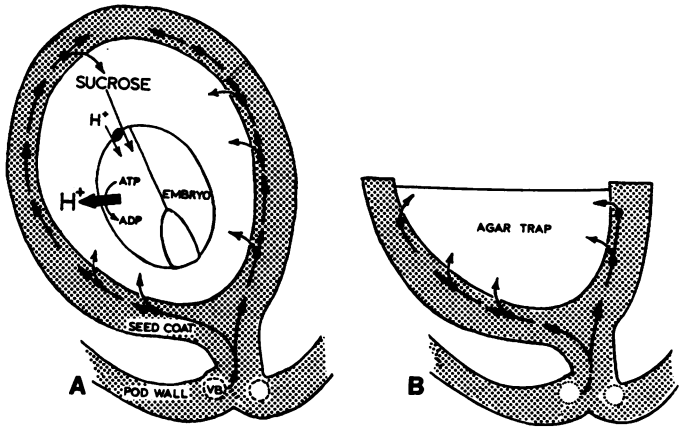

FIG. 2. Schematic illustration of intact and surgically altered soybean seeds importing and unloading photosynthate. A, Transverse section through an entire seed attached via the funiculus to the pod placenta and/or vascular bundles. Arrows in the seed coat represent the route of imported photosynthate. Circles on these arrows schematically represent some, but not all, sites of phloem unloading. Within the cotyledons of the embryo, the large arrow represents a membrane-bound ATPase responsible for electrogenic proton extrusion to the freespace. Sucrose/proton cotransport is represented by the smaller arrows on the embryo. B, Embryo removed from the surgically altered seed coat and replaced with agar or other trap matrix. Symbols remain the same as in 1A.

Table I. *Effects of Surgery on ^{14}C Import by Adjacent Seeds 2.5 Hours after Labeling the Plant with 50 μCi $^{14}CO_2$*

Seed Component	^{14}C Content of Tissue	
	Assayed[a]	Total[b]
	dpm	
Entire seed		
Embryo	66,000	192,000
Seed coat	126,000	
Half-seed control		
Half embryo	30,000	184,000
Remaining seed coat	62,000	
Agar-filled system		
Agar	32,000	180,000
Remaining seed coat	58,000	

[a] Values represent the means of four determinations in two experiments. Relative seed position within the pod was unimportant.

[b] Tissue dpm was totaled in the case of the entire seed or doubled in altered seeds to estimate import on a total seed basis, assuming that altered seeds had been cut exactly in half.

tated removal of older embryos. The exposed inner (endothelial) surface of the seed coat (12) formed a 'cup' (Fig. 1) which was routinely pretreated for 10 to 30 min with various aqueous treatments (inhibitors, buffers, hormones, salts, etc.) before replacement with an appropriate trapping solution. Agar (4%), when added as a warm solution (35–40°C) to within 1 mm of the seed coat cut edge (Fig. 2), quickly solidifies to form a replica of the removed half embryo. This provides a convenient solute trap, in contact with the entire inner surface of the seed coat. In some cases, EDTA, EGTA[2], $CaCl_2$, or other treatments were incorporated in the agar. The adjacent half-seed (with its half-embryo)

[2] Abbreviations: EGTA, ethyleneglycol-bis(β-amino-ethyl ether)-*N,N'*-tetraacetic acid; PCMBS, *p*-chloromercuribenzene sulfonate; NaF, sodium fluoride; $NaAsO_2$, sodium arsenite; DNP, 2,4-dinitrophenol.

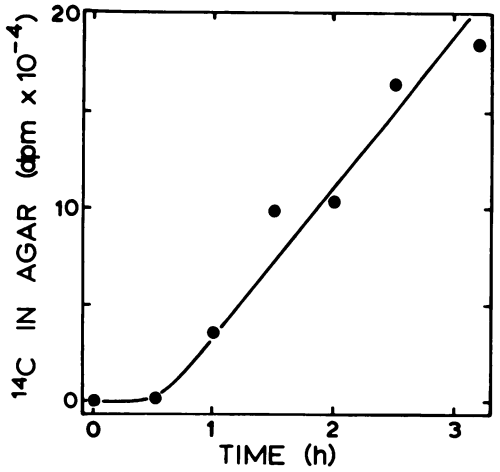

FIG. 3. Time course of ^{14}C-photosynthate accumulation in the agar trap matrix following labeling (t = 0) of the entire plant with 250 μCi $^{14}CO_2$. Values represent the means of four replicate fruit, each with two altered seeds.

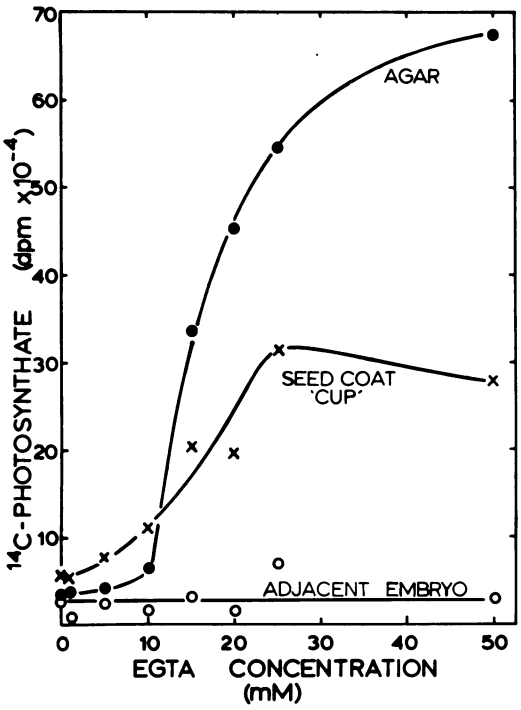

FIG. 4. Effects of EGTA incorporation into the agar trap matrix on: (×), the import of label by agar-filled seed coat cups; and (●), subsequent accumulation in that agar. Adjacent half-embryos (○) provided controls (no EGTA) for comparison. Values represent the mean ^{14}C accumulated in four replicate fruit, 2.5 h after labeling the plant with 100 μCi $^{14}CO_2$.

provided a control for most experiments, regardless of its position within the pod (data not shown). To prevent the possibility of phloem bleeding from the cut ends of the vascular bundles, an effort was made to prevent physical contact of the agar with the cut seed coat edge (Fig. 2). Pod incisions were wrapped with prestretched parafilm to maintain high humidity within the pods.

Plants with several altered pods (parafilm-wrapped) were enclosed in a large acrylic chamber and labeled for 15 min with $^{14}CO_2$ as described previously (13) and in the figure and table legends. Chase periods of various durations (typically 2–3 h) were in air under light-saturating conditions (1,000 μE/m^2·s, xenonarc lamp; Atlas Electric Devices Co., Chicago, IL). In other experiments, [^{14}C]sucrose was applied to an abraded leaf (2) and the

translocated label trapped in the agar of altered, subtending fruit. Pods were subsequently harvested and held on ice as the agar was removed for analysis. Extraction and analysis of embryo, seed coat, and agar solutes was as previously described (14), with the following exceptions: analyses of amino acids were performed in lithium citrate on a Beckman 119CL amino acid analyzer, and ureides were determined with ion-exchange HPLC with 0.01 N H_2SO_4 solvent and refractive index detection (Rainbird and Thorne, unpublished). We conducted similar experiments on lupin (*Lupinus albus* cv Ultra) and obtained nearly identical results; therefore, only data for soybean will be presented.

RESULTS AND DISCUSSION

Unloading of photosynthate destined for the developing embryo occurs from phloem within the maternal seed coat (11, 12). Little is known about the nature of the transport mechanism, or membrane permeability changes, which facilitate sucrose exit from the sieve element/companion cell complex. However, the presence of large companion cells connected via plasmodesmata to the seed-coat sieve tubes (11) and the sensitivity of ^{14}C-photosynthate import by soybean seeds to anoxia and low temperature (13) provide circumstantial evidence for a direct or indirect metabolic component. Unloaded sucrose apparently moves intercellularly to the free space separating the seed coat and the embryo (11, 12).

Using the present technique, careful removal of the embryo renders the sites of unloading accessible via the free space of the seed coat cup. Phloem unloading was not impaired by cutting, embryo removal, or replacement with agar, for kinetics of ^{14}C-photosynthate import were similar to published (11) values for intact fruit (data not shown) and were generally similar for both agar and half embryos (Table I). The similarity of the agar and control-embryo ^{14}C levels suggests that phloem bleeding from the cut ends of the seed coat vascular bundles did not occur. Apparently, the agar simulated the low-sucrose conditions maintained in the free space *in vivo* by the transport activities of the embryo (9, 14).

Accumulation of ^{14}C-photosynthate in the trap matrix was nearly linear with time after arrival of label in the fruit (Fig. 3), and was maintained at rates of 0.5 to 1.0 μmol/h for up to 8 h in experiments in which buffer (5 mM Mes, pH 6.0, containing 100 mM mannitol and 1 mM $CaCl_2$) replaced agar as the solute trap (data not shown). Sucrose generally comprised over 95% of the carbohydrate and 90% of the total C imported, but the hexoses, glucose and fructose, comprised 5 to 20% in some experiments in which buffer was the solute trap (data not shown). Amino acids (primarily glutamine) and ureides provided the remaining C (data not shown). The C/N ratio of the trapped solutes (31 mg C/mg N) was consistent with published analyses of fruit phloem sap (8). Fruit growth and C/N budgeting studies confirmed the physiologic validity of the trapped phloem sap; C and N solute delivery was fully adequate to support the observed seed growth and C/N increments (Rainbird and Thorne, unpublished).

Normally, the kinetics of ^{14}C import dictate that the seed coat will be more heavily labeled than the agar or embryo until 4 to 5 h after arrival of ^{14}C-photosynthate in the fruit (data not shown). However, the presence of greater than 5 mM EGTA or EDTA in the agar (pH 6.0) greatly enhanced total import (Fig. 4). EGTA was somewhat more effective that EDTA (data not shown). Under these conditions, the agar generally contained more label than the seed coat after only a 2-h period. The EGTA-enhanced import of ^{14}C could be 70% reversed by incorporation of $CaCl_2$ (10 mM EGTA, 100 mM $CaCl_2$) in the agar (data not shown). Import of ^{14}C-photosynthate in the absence of EGTA was insensitive to Ca^{2+} (data not shown). These data suggest that enhanced membrane permeability in vascular tissues, perhaps through chelation of membrane-bound divalent cations (1, 7), was responsible for increased ^{14}C import. Physiological unloading was apparently overwhelmed by massive phloem leakage or exudation in the presence of EGTA (Fig. 4).

EGTA provided a mechanism for greatly stimulating the import and, thus, 'sink strength' of a seed to determine its effects on the unloading in adjacent seeds sharing vascular connections in the same pod. When 25 mM EGTA was present in the agar of the seed coat cup in one seed position, however, the 30-fold stimulation of import there had absolutely no effect on the unloading and release of ^{14}C-photosynthate to controls in other seed positions, regardless of location in the pod or total seed number (data not shown). These data are consistent with transport autonomy among seeds (at least on a short-term basis); interactions may be obscured by the high sucrose concentrations in the phloem relative to that of the seed coat.

Diethylstilbestrol (1–5 mM) also stimulated photosynthate import in a manner that was insensitive to divalent metal ions (data not shown).

To characterize further the unloading mechanism, we briefly

Table II. *Inhibition of [^{14}C]Sucrose Unloading in Soybean Seed Coats and Release to Agar Traps*

Seed Coat Cup Pretreatment	Trap Matrix (pH 6)	^{14}C-Photosynthate[a]		Inhibition of ^{14}C Unloading
		Agar	Cup	
		dpm		%
None	Agar	7,000	213,000	
Water[b]	Agar	7,000	225,000	
PCMBS[c]	Agar	1,800	292,000	75
PCMBS, DTT[d]	Agar	4,000	174,000	43
Water[b]	Agar + 15 mM EGTA	119,000	90,000	
PCMBS[c]	Agar + 15 mM EGTA	600	8,000	99
PCMBS, DTT[d]	Agar + 15 mM EGTA	17,000	40,000	86
None	Agar + 5 mM NaF	60	70	100
None	Agar + 5 mM $NaAsO_2$	40	30	100
None	Agar + 1 mM DNP	80	50	100

[a] Import of ^{14}C-photosynthate during a 2-h period following labeling of plants with 50 μCi $^{14}CO_2$. Each value represents the mean of four replicates in two experiments.

[b] Thirty-min water wash, blot, agar added to cup.

[c] Ten min 2.5 mM PCMBS, 10 min water wash, blot, agar added to cup.

[d] Ten min PCMBS, 10 min water wash, 10 min 25 mM DTT, blot, agar added to cup.

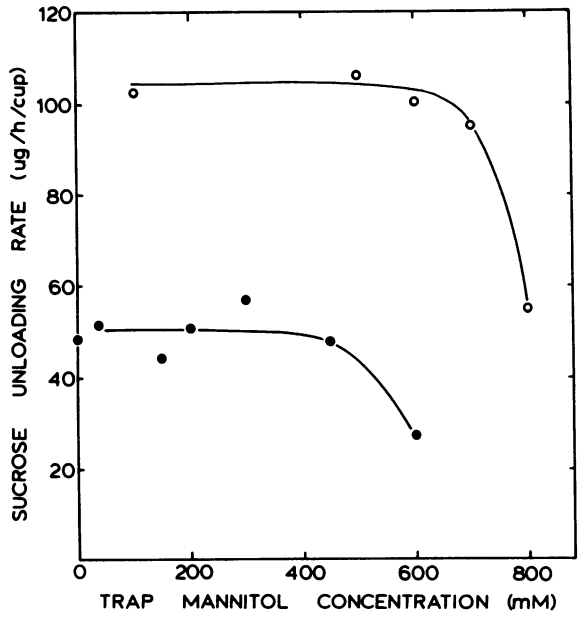

FIG. 5. Rate of phloem unloading (μg sucrose/h·seed cup) as a function of osmotica and PAR: (○), 1,200 μE/m²·s; and (●), 300 μE/m²·s at canopy level. Mannitol solutions contained 1 mM $CaCl_2$ and 25 mM Mes (pH 6.0) and were replaced in the cups at 30-min intervals. Sucrose was determined with HPLC. Values represent the means of 4 (●) or 8 (○) determinations in three experiments.

pretreated newly-exposed seed coat cups with PCMBS (2.5 mM, pH 6.0. 10 min, followed by a 10-min water wash) and then added agar with or without 15 mM EGTA. PCMBS, a nonpenetrating sulfhydryl reagent known to bind reversibly to membrane-bound sucrose carriers (2, 9, 14), markedly inhibited sucrose release to the agar during the 3-h period following PCMBS exposure and $^{14}CO_2$ labeling (Table II). In the absence of EGTA in the agar (Table II, top portion), it is significant that PCMBS pretreatment did not affect phloem transport of ^{14}C into the seed coat tissue but effectively inhibited its release to the agar trap (75%). Although several interpretations are possible, we conclude that a brief PCMBS pretreatment of the seed coat apoplast effectively prevented subsequent sucrose unloading from the phloem. Exposed sulfhydryl groups on essential membrane proteins, perhaps sucrose carriers, were seemingly affected. Support for this conclusion is derived from the partial reversal of PCMBS inhibition by DTT applied to the seed coat cup after PCMBS treatment but before agar was added (Table II).

The presence of 15 mM EGTA in the agar greatly enhanced ^{14}C import in the controls, but also greatly potentiated PCMBS inhibition of both import in the seed coat and release of ^{14}C to the agar. Under these conditions, DTT was much less effective in reversing the inhibition (Table II, bottom portion). We have no satisfactory explanation for these observations.

Treatment with the penetrating metabolic inhibitors NaF, $NaAsO_2$, or DNP completely prevented both ^{14}C import in the seed coat and subsequent release to the agar (Table II). This is apparently much different than the PCMBS mechanism of inhibition and suggests disruption of structural and functional integrity of the vascular bundles of the seed coat cup, similar to the effects of cyanide on phloem translocation (3).

Unloading of sucrose into agar (containing 25 mM Pipes, pH 6) is stimulated 350 to 400% by 20 μM fusicoccin and 80 to 100% by dilute KCl (2 to 20 mM). NaCl is less effective than KCl (unpublished data). These and other data suggest the involvement of an ion-mediated sucrose transport mechanism.

Substituting various mannitol solutions for agar as a solute trap allowed rapid HPLC analysis of unloaded photosynthate. Visible seed coat cup tissue plasmolyzed in the presence of 200 mM mannitol or sucrose (data not shown), but phloem unloading was unaffected by <500 mM mannitol (Fig. 5). Rates of unloading were clearly dependent on the photosynthetic rate of the plant; well-watered plants held under light-saturating conditions maintained the highest rates of unloading (Fig. 5).

In conclusion, this is a convenient and apparently valid technique for studying the mechanism of phloem unloading *in vivo*. With this technique, we have determined that transport of photosynthate into the seed coat or exit to the freespace is extremely sensitive to treatments which may alter membrane integrity and permeability (inhibitors of energy metabolism and cation chelators), and apparently involves essential sulfhydryl groups on membrane-bound proteins.

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LITERATURE CITED

1. COSTELLO LR, JA BASSHAM, M CALVIN 1982 Enhancement of phloem exudation from *Fraxinus uhdei* Wenz (Evergreen Ash) using ethylenediaminetetraacetic acid. Plant Physiol 69: 77–82
2. GIAQUINTA T 1976 Evidence for phloem loading from the apoplast. Chemical modification of membrane sulfhydryl groups. Plant Physiol 57: 872–875
3. GIAQUINTA RT 1977 Mechanism of cyanide inhibition of phloem translocation. Plant Physiol 59: 178–180
4. GIAQUINTA RT 1980 Translocation of sucrose and oligosaccharides. *In* J Preiss, ed, Biochemistry of Plants, Vol 3. Academic Press, New York, pp 712–319
5. GIFFORD RM, LT EVANS 1981 Photosynthesis, carbon partitioning, and yield. Annu Rev Plant Physiol 32: 485–509
6. GLASZIOU KT, KR GAYLER 1972 Sugar accumulation in sugarcane. Role of cell walls in sucrose transport. Plant Physiol 49: 912–913
7. KING RW, JAD ZEEVAART 1974 Enhancement of phloem exudation from cut petioles by chelating agents. Plant Physiol 53: 96–103
8. LAYZELL DB, TA LARUE 1982 Modeling C and N transport to developing soybean fruits. Plant Physiol 70: 1290–1298
9. LICHTNER FT, RM SPANSWICK 1981 Electrogenic sucrose transport in developing soybean cotyledons. Plant Physiol 67: 869–874
10. TANNER W 1980 On the possible role of ABA on phloem unloading. Ber Dtsch Bot Ges Bd 93: S 349–351
11. THORNE JH 1980 Kinetics of ^{14}C-photosynthate uptake by developing soybean fruit. Plant Physiol 65: 975–979
12. THORNE JH 1981 Morphology and ultrastructure of maternal seed tissues of soybean in relation to the import of photosynthate. Plant Physiol 67: 1016–1025
13. THORNE JH 1982 Temperature and oxygen effects on ^{14}C-photosynthate unloading and accumulation in developing soybean seeds. Plant Physiol 69: 48–53
14. THORNE JH 1982 Characterization of the active sucrose transport system of immature soybean embryos. Plant Physiol 70: 953–958
15. THORNE JH, RT GIAQUINTA 1983 Pathways and mechanisms associated with carbohydrate translocation in plants. Symp Soc Exp Bot. In press
16. WOLSWINKEL P 1974 Enhanced rate of ^{14}C-solute release to the free space by the phloem of *Vicia faba* stems parasitized by *Cuscuta*. Acta Bot Neer 23: 177–188