RESEARCH PAPER

Dynamics and control of phloem loading of indole-3-acetic acid in seedling cotyledons of *Ricinus communis*

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Received 17 March 2016; Accepted 6 June 2016

Editor: Angus Murphy, University of Maryland

Abstract

During seed germination, sugars and auxin are produced from stored precursors or conjugates respectively, and transported to the seedling axis. To elucidate the mode of travel of indole-3-acetic acid (IAA) into the phloem, a solution of [3H]IAA, together with [14C]sucrose, was injected into the endosperm cavity harboring the cotyledons of germinating seedlings of *Ricinus communis*. Phloem exudate from the cut hypocotyl was collected and the radioactivity recorded. Sucrose loading into the phloem was inhibited at higher IAA levels, and the rate of filling of the transient pool(s) was reduced by IAA. IAA was detected within 10 min, with the concentration increasing over 30 min and reaching a steady-state by 60 min. The kinetics indicated that phloem loading of IAA involving both an active, carrier-based, and a passive, diffusion-based component, with IAA traveling along a pathway containing an intermediary pool, possibly the protoplasts of mesophyll cells. Phloem loading of IAA was altered by sucrose, K+, and a range of non-specific and IAA-specific analogs and inhibitors in a manner that showed that IAA moves into the phloem from the extra cotyledonary solution by multiple pathways, with a carrier-mediated pathway playing a principal role.

Key words: Cotyledons, germination, indole-3-acetic acid, loading pathways, NAA, PCMB, PCMBS, phloem loading, *Ricinus*, sucrose, TIBA.

Introduction

Seeds contain not only food reserves in the form of oils or starch but also hormones in a conjugate or precursor form (Normanly et al., 2010). In the case of the auxin [indole-3-acetic acid (IAA)], indole-3-acetyl aspartate, IAA inositol or glycoside conjugates, or complexes thereof, or peptide conjugates can be found in the endosperm or cotyledons, as exemplified by maize (*Zea mays*) and bean (*Phaseolus vulgaris*) (Normanly et al., 2010). During germination, the oil or starch reserves are converted into sucrose for transport to the developing embryo, whereas IAA is released from conjugates (Morris et al., 2010; Normanly et al., 2010). The mechanism by which IAA enters the transport stream is currently unknown. Germinating seeds of castor bean (*Ricinus communis*) form an ideal model system to address this question because the reserves are held in the endosperm and during germination the solubilized reserves are taken up by leaf-like cotyledons located in the center of the endosperm tissues. These cotyledons function initially as absorptive organs, and only later emerge to function as the first leaves of the seedling. As these cotyledons have no cellular connection to the endosperm reserves, both reserve materials and hormones alike must be solubilized, transported into the peri-cotyledonary space, and then taken up by the cotyledons prior to transport to the rest of the seedling. Cotyledons of the
**Ricinus** seedling readily take up solutes from the incubation medium via the whole blade surface (Komor et al., 1991) and, when the hypocotyl is severed, the seedlings exude phloem sap from the cut hypocotyl. The imbibed, germinating seedlings of castor bean are large enough for the easy application of tracer materials and pharmaceutical modifying agents into this peri-cotyledonary space, so that germinating **Ricinus** seedlings make an excellent system to address these questions. In addition, **Ricinus** seedlings have been extensively utilized for studies on sucrose uptake by the cotyledons, so the characteristics of sucrose uptake are well known (Komor et al., 1991; Orlich and Komor, 1992; Orlich et al., 1998).

**Sucrose uptake by the phloem in **Ricinus** seedlings**

During germination and the first few days of growth, sucrose, derived from stored materials in the endosperm, is absorbed by the cotyledons from the surrounding medium and thence transported into the phloem (Weig and Komor, 1996). Sucrose is the major solute in the phloem (Komor et al., 1991) and there is an accumulation of sucrose in the phloem to a concentration of 270 mM, exceeding that in the surrounding medium, indicative of active transport (Kallarackal et al., 1989). At least 50% of sucrose is loaded into the apoplastic pathway without involvement of the symplastic route (Komor et al., 1991). The pH dependence of sucrose uptake (with an optimum of pH 5) (Weig and Komor, 1996), combined with the alkalization of the apoplasm and membrane depolarization of mesophyll cells, is indicative of H⁺-sucrose co-transport (Koehler et al., 1991; Weig and Komor, 1996).

There is also a direct uptake of sucrose by the sieve tube–companion cell complex from the apoplastic (Weig and Komor, 1996). About half of the sucrose exported is loaded into the phloem directly from the apoplasm, while the other half takes the route via the mesophyll. Mesophyll-derived sucrose is released into the apoplast adjacent to the phloem prior to loading into the phloem (Orlich and Komor, 1992), so that loading into the phloem is by both a direct and an indirect apoplastic pathway, as well as symplasmic loading.

Williams et al. (1990), using cotyledon-derived plasma membrane vesicles, provided strong evidence for a sucrose–proton symporter system in the plasma membrane of cells of **Ricinus** cotyledons. Sucrose uptake had a $K_m$ of 0.87 mM (Komor et al., 1991), was stimulated by a pH gradient with a pH optimum of pH 6.5, was inhibited by vanadate, the sulfhydryl reagent p-chloromercuribenzenesulfonate (PCMSB), and the protonophore CCCP, and showed strong specificity for ATP as a substrate (Williams et al., 1990). A sucrose carrier gene was found to be expressed in the cotyledons of **Ricinus** seedlings at a similar level at germination and 3–6 d after germination, with the greatest expression in the lower epidermal layer and the phloem, consistent with an active loading role for these cells (Bick et al., 1998a).

The energy source for the process of phloem loading in those plants utilizing sucrose–proton co-transport is an electrochemical potential gradient maintained through the active extrusion of protons by H⁺ pumps into the apoplastic free space (Hutchings, 1978a). In excised cotyledons of **Ricinus** seedlings, externally applied sucrose readily enters the apoplast, whence it is actively loaded into the sieve element–companion cell complex by an H⁺–sucrose co-transport system (Hutchings, 1978a). A significant portion of applied sucrose may, however, be taken up by the mesophyll and passed on to the phloem via symplastic flow (Orlich et al., 1998). Sucrose uptake by excised **Ricinus** or soybean cotyledons shows a biphasic response to sucrose concentration. At low external levels, sucrose uptake operates as a high-affinity, carrier-based process, characterized by low rates of H⁺ and sucrose influx. With increasing sucrose concentration, a linear, diffusion-like phase becomes predominant between 20 mM and 50 mM sucrose, showing a diminished dependence on net H⁺ influx and a consequent sharp decline in the stoichiometry of H⁺:sucrose (Kriedemann and Beevers, 1967; Komor, 1977; Hutchings, 1978a; Delrot and Bonnemain, 1981; Lichtner and Spanswick, 1981).

When potassium ions are included along with sucrose in the incubation medium, there is a bimodal effect on sucrose loading depending on K⁺ concentration: stimulation of loading at the lower range (generally below 10 mM K⁺), and inhibition at higher levels (Komor et al., 1977; Hutchings, 1978b; Van Bel and Koops, 1985; Schobert et al., 1998). At the lower range, K⁺ influx will allow a modest rate of discharge of the pH gradient, which results in a faster recirculation of H⁺ and enhanced sucrose loading without a major drop in membrane potential. At increasingly higher levels, K⁺ influx will depolarize the plasma membrane, causing a concentration-dependent decrease in transport activity.

Amino acids are also taken up by proton-mediated carriers. Glutamine was taken up by plasma membrane vesicles with a $K_m$ of 0.35 mM and a sensitivity to both PCMSB and CCCP similar to that of sucrose (Williams et al., 1990). Bick et al. (1998a) found genes for two putative amino acid carriers to be abundantly expressed in the cotyledons.

**Long-distance auxin transport**

Auxin is transported in the plant by two main mechanisms, namely cell to cell transport, which is responsible for the developmental regulation via the spatial distribution of auxin, and long-distance transport away from the source tissues by an unregulated bulk flow in the mature phloem (Petrášek and Friml, 2009). Auxin is naturally exported from source leaves in the phloem (Baker, 2000a; Morris et al., 2010). **Ricinus** phloem sap, collected via incisions into the inner bark, has been found to contain 13 ng ml⁻¹ IAA (as analyzed by GC-MS) (Baker, 2000b), and this sap provides one of two sources of auxin to the rest of the plant, the other being cell to cell polar auxin transport; the xylem had only a small fraction of this amount (Baker, 2000b). IAA applied to mature pea leaflets was initially exported via the phloem as detected by aphids feeding on the stem or recovery in exudates collected from severed petioles (Cambridge and Morris, 1996), and endogenously produced IAA was found in the phloem exudate from excised pea leaflets at a production rate of 7.7 pg leaflet⁻¹ h⁻¹ (Jager et al., 2007), though as the volume was not recorded the concentration cannot be calculated. Mature leaves are therefore one source of the IAA in the basipetal transport.
stream. After a period of hours, applied IAA exported from leaves in the phloem was found transferred into the extravascular polar auxin transport pathway, though reciprocal transfer from the polar auxin transport stream into the phloem probably does not occur (Cambridge and Morris, 1996).

Polar auxin transport relies on a pH gradient-driven weak acid passive uptake and carrier-mediated uptake, combined with specific IAA efflux carriers located in the base of each cell (Petrášek and Friml, 2009; Morris et al., 2010), leading to an iteration from cell to cell and thus transport in a basipetal direction. IAA loaded into the phloem will be subject to the factors that influence phloem translocation (Morris et al., 2010). A principal such factor is sucrose (or other transported carbohydrate) loading into the phloem. Several pathways of phloem loading exist, namely simple concentration-mediated diffusion, transmembrane proton co-transport, and a polymer trap mechanism, which may operate singly or in combination, and for sugars this varies with the species (Rennie and Turgeon, 2009). The mechanism of IAA entry into the phloem is unknown. Previous work on IAA uptake into stem segments has revealed various mechanisms of entry into the transport stream, and the way that these can be distinguished pharmacologically (Davies and Rubery, 1978). The aim of this work was to ascertain the mechanism and extent of IAA transport from the seed source into the phloem of Ricinus seedlings. Ricinus seedling cotyledons represent a natural way of investigating this phenomenon because there is a lack of cellular connection between the endosperm and the cotyledons (Komor et al., 1991) such that the application of sucrose and IAA mimics the natural pathway that already exists. This pathway into the cotyledonary cells and on into the phloem can be elucidated for IAA using techniques similar to those already used to examine sucrose movement for extra-cotyledonary sucrose into the phloem of the Ricinus seedlings.

Radioactivity levels were generally in the range of 0.5–1.5 MBq m⁻¹ for [³H]IAA and 0.2–0.7 MBq m⁻¹ for [¹⁴C]sucrose, with corresponding concentrations averaging ~0.8–1.6 µM IAA and 8 µM sucrose. In some experiments, the concentration of IAA was adjusted to several different levels in a set of IM preparations by adding non-radioactive IAA, while keeping their [³H]IAA content the same. Comparable experiments were also done with sucrose. Actual radioactivity and concentration levels, together with notes on other components (e.g. specific chemical probes), if any, are provided with the individual figures. [³H]IAA (925 GBq mmol⁻¹) was purchased from American Radiolabeled Chemicals (ARC; St Louis, MO, USA); [¹⁴C]sucrose was obtained from Sigma (St Louis, MO, USA) (20.9 GBq mmol⁻¹) or from ARC (22.9 GBq mmol⁻¹).

**Incubation buffer (IB)**
IB was used to incubate the endosperm during the transport experiment. It contained the same buffer as that present in the corresponding IM injected within the endosperm, but not including any radiolabeled substances or chemical probes.

**Injection, transport, and recovery of radiolabeled substances**
In preparing the seedlings for injection, the hypocotyl was cut with a sharp razor blade to remove the roots and lower hypocotyl, thus leaving a hypocotyl stump, 7–10 mm in length, attached to the cotyledons enclosed within the endosperm. Using a microsyringe, 5 µl of IM was injected into the endosperm cavity between the cotyledons, thus exposing the two enclosed cotyledons to the radiolabeled substances being tested (Fig. 1). Then, the endosperm was placed in a small beaker, between layers of absorbent paper moistened with IB, ensuring that the endosperm with the emerging hypocotyl stump was held in an upright position. Freely exuding phloem sap from the cut surface of the hypocotyl was collected during 10 min intervals, starting at 0–10 min, generally for an hour, with a graduated microcapillary tube resting on the hypocotyl stump. High relative humidity was maintained throughout the transport period. The volume of the collected exudate was recorded, and the sample was transferred with 95% ethanol as a rinse into a liquid scintillation vial for analysis. All operations were carried out under fluorescent laboratory lighting at ~15 µmol m⁻² s⁻¹.

**Radioactivity counting and data presentation**
The ³H and ¹⁴C activity in each exudate sample was determined simultaneously using Ecoscint (National Diagnostics, Atlanta, GA, USA) with a Beckman (Fullerton, CA, USA) LS 1801 liquid scintillation counter programmed for dual-isotope DPM analysis. From the radioactivity of each isotope, the concentration of the respective transported substance was calculated. Specific activities used for the conversion were generally those provided by the manufacturer (with

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**Materials and methods**

**Plant material**
Castor bean (Ricinus communis L. cv. Sanguineus) seeds (Stokes Seeds, Inc., Buffalo, NY, USA) were sown in a soil-less medium ‘Cornell Mix’ (Boodley and Sheldrake, 1982), composed of peat, vermiculite, and perlite at a ratio of 3:2:1, with supplements of ferti-lizers and pulverized dolomitic limestone. Seedlings were raised in a vermiculite, and perlite at a ratio of 3:2:1, with supplements of ferti-

**Injection medium (IM)**
Simultaneous transport of radiolabeled IAA and sucrose IAA and sucrose transport were directly compared in individual seedlings using an IM in which defined levels of [³H]IAA and [¹⁴C] sucrose were combined in 30 mM MES buffer at pH 6.3. Either KOH or NaOH was used for pH adjustment in the MES stocks, giving a terminal concentration of 20 mM K⁺ or Na⁺ in the IM.

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**Fig. 1.** Diagram of the treatment method. The hypocotyl of hydrated 6-day-old Ricinus seedlings was cut off, leaving a 1 cm stump, which was placed against a calibrated 10 µl capillary tube. A 5 µl aliquot of injection medium containing the radiolabeled substances to be transported was injected into the endosperm cavity between the cotyledons. The capillary tube with exuded phloem sap was replaced every 10 min, the volume recorded, and the contents counted for radioactivity.
activities of [\(^{1}\)H]IAA corrected for decay). Specific activity values were recalculated for those IM preparations in which a radiolabeled substance was supplemented with the corresponding non-radioactive compound. For the purpose of data evaluation and presentation, the concentration of a transported substance in each sample was expressed as a percentage of the concentration in the corresponding IM preparation; the data represent each individual 10 min collection and are not cumulative. The variable \(t_{0.5}\) is equal to the time, in minutes, required for the transported substance to attain or approach one half of the steady-state level in the phloem exudate, or one half of the highest concentration obtained within a transport period of 1 h in case a steady state had not been fully attained. For plots not showing a tendency toward a steady state, and/or remaining at or near linearity throughout the transport period, the \(t_{0.5}\) value is marked with an asterisk, indicating a failure to attain a steady state; the value of \(t_{0.5}\) is then based on one half of the highest level attained within 1 h of transport.

**Statistical analysis**

All of the data presented here are the combined results of at least two independent experiments, and represent the means of at least five replicate measurements. The number of replicates and of repeated experiments is stated in each figure legend. Data analysis was done with the Windows-based statistical system Minitab (Minitab, Inc.). To test the significance of each treatment effect compared with the relevant control, we calculated their \(F\)-ratio along with the corresponding probability value using analysis of covariance (ANCOVA), a model that allows for the correction of variation due to selected experimental conditions (e.g. sampling time, developmental variation, etc.) as appropriate. Significant treatment effects, thus corrected, were those within the probability range of \(P=0.00–0.05\).

**Results and Discussion**

**Simultaneous transport of differentially labeled IAA and sucrose in phloem: effect of IAA concentration**

After a buffered medium containing both [\(^{1}\)H]IAA and \([^{14}\)C] sucrose was injected in the endosperm cavity harboring the cotyledons, both substances were recovered in the phloem exudate from the severed end of the hypocotyl stump ~1 cm below the cotyledons and endosperm (Fig. 1). We consider this a reasonable representation of the likely uptake and transport of endogenous IAA and sucrose, as the endogenous compounds must follow the same pathway and the experimental concentrations used were within the physiological range. The identification of phloem as the transporting tissue has been provided by Kallarackal et al. (1989), including the high concentration of sucrose in the sap, inhibition by calcium chloride, and the lack of an apoplastic marker in the sap. A broad range of IAA concentrations from 0.0016 mM to 20 mM was tested, all with identical [\(^{1}\)H]IAA content. Freely exuding phloem sap was collected at 10 min intervals up to 1 h. The volume exuded in 10 min ranged from 2 ml to 6 ml. The volume and amount of radioactivity recovered were highly variable, from seedling to seedling, though largely consistent over an hour within any one seedling. According to Komor et al. (1991), the flow rate is determined by the rate of phloem loading, the osmotic water uptake, the resistance of the sieve tubes, and the percentage of open phloem; factors that account for variability of the exudation rate include the seedling age and stage of development, handling and quality of cut, and the composition of the medium. Despite differences in the exudation rate, the solute composition and concentration of the exudate are reported to be very similar between individual seedlings (Komor et al., 1991). When our results were corrected for the volume of solution recovered, namely as the concentration (in %) relative to the injected concentration, patterns became clear. At all applied levels, IAA was detected within the first 10 min, and the relative concentration of IAA appearing in the exudate increased at similar rates for the first 30 min (Fig. 2A). Over the next 30 min, the IAA exudation rate gradually came to an apparent steady-state level. The steady-state concentration of radiolabeled IAA in the phloem exudate was ~0.01 \(\mu\)M. The fact that the IAA appears rapidly in the phloem sap, the loading is via a cotyledon (a leaf homolog), and has a pattern similar to sucrose, leads us to conclude that the measured IAA reflects transport in the phloem, though some contribution of cell-to-cell movement through the hypocotyl transport remains a possibility over longer periods (Petrášek and Friml, 2009).

The observed pattern of accumulation in the phloem exudate may be obtained when the applied radiolabeled substance passes through one or more transient pools of defined size in the transport pathway to the site of phloem loading. When the substance entering the phloem has reached a steady fraction of the concentration in the IM, the recovered concentration also attains a steady state. The fact that the relative steady-state levels for different IAA applied concentrations were in close proximity indicates that IAA loading in the phloem has stabilized at very different IAA levels—stretching over four orders of magnitude—roughly in proportion to the applied concentration, except that it was somewhat less at the highest concentration (20 mM). These observations show that IAA transport in phloem can occur efficiently over a very wide concentration range, reflecting perhaps the activity of a complex transport system with multiphasic kinetics (Komor et al., 1977). More specifically, the results suggest the operation of a diffusive, ‘linear’ component at the higher levels (Komor et al., 1977; Lichtner and Spanswick, 1981), analogous perhaps to the mode of sucrose transport at varying sucrose concentrations (Fig. 6B). The fact that the relative IAA transport rate at the highest level of applied IAA (20 mM) was somewhat less than linear (Fig. 2A) suggests that IAA concentration is not the sole factor controlling the loading process.

The \([^{14}\)C] sucrose was injected into the endosperm cavity at a sufficiently low level (0.35 mM) such that it would have a negligible effect on the native sucrose concentration, estimated to be ~90 mM (Kriedemann and Bevers, 1967). The transported \([^{14}\)C] sucrose concentration in the phloem exudate at 1 h, expressed as the percentage of the injected sucrose concentration, was ~4% at the two lower applied IAA levels (Fig. 2B). The corresponding transported sucrose concentration was only ~2.5% in the presence of 2.0 mM or 20.0 mM IAA, indicating that sucrose loading in the phloem is slightly subject to inhibition at higher, non-physiological IAA levels. As the time-dependent changes in transported sucrose concentration revealed, the trend was at or near linearity for the lowest applied IAA level throughout the 1 h run [in the figures, the \(t_{0.5}\) value (the time in minutes required for the transported substance to attain the half steady-state level
in the phloem exudate) is marked with an asterisk to note that a steady-state has not been attained. However, the trend became progressively more sigmoid with increasing IAA levels: at 20 μM IAA, there was in the first 20 min a much slower appearance of the labeled sucrose in the exudate, indicating that the rate of filling of the transient pool(s) was reduced by IAA. Perhaps as a consequence, sucrose transport at 20 μM applied IAA came to a steady state at a concentration much below that of the lowest IAA level (Fig. 2B).

NAA competes with IAA transport in the phloem

The synthetic auxin α-naphthaleneacetic acid (NAA) is analogous to IAA in many of its physiological properties, including the ability to serve as a competitive substrate for the auxin efflux carriers. However, in contrast to IAA, NAA has only marginal affinity for the auxin influx carrier (Delbarre et al., 1996; Marchant et al., 1999). Externally applied NAA enters cells by diffusion. On the basis of these attributes, we selected NAA as a diagnostic probe to test whether IAA loading into the phloem requires auxin efflux carrier activity. We measured the transport of [3H]IAA (0.78 μM) injected in combination with NAA at concentrations of 0.0, 0.1, 1.0, and 10.0 mM. Analysis of the collected samples of phloem exudate showed that [3H]IAA transport was inhibited in plants treated by NAA, the effect being most highly expressed at both 0.1 mM and 10.0 mM (Fig. 3A). In the affected plants, progress toward a steady state was slower, and it was reached at a lower IAA concentration. The competitive effect of NAA suggests that in Ricinus cotyledons the passage of [3H]IAA through the loading pathway is facilitated by auxin efflux carriers. Basipetal transport of IAA, involving efflux carriers, may take place in files of parenchyma cells that are closely associated with minor veins in developing leaves, as described in the next section (Mattsson et al., 1999, 2003; Aloni, 2010).

To test whether sucrose transport may also be altered in the presence of NAA, [14C]sucrose at 8.3 μM was included in the IM along with [3H]IAA. The effect, compared with that on IAA transport, was much less clear. There was some reduction in sucrose transport but only at the lowest (0.1 mM) level of NAA (Fig. 3B). This is an interesting result as it seems to contradict the inhibitory effect of IAA on sucrose transport (Fig. 2B).

Phloem loading of IAA is stimulated by the auxin transport inhibitor triiodobenzoic acid

Phloem transport of [3H]IAA from cotyledons of Ricinus seedlings was stimulated at both 20 μM and 100 μM 2,3,5-triiodobenzoic acid (TIBA) (Fig. 4A). The results suggest that a TIBA-enhanced IAA accumulation in auxin-transporting tissues caused a diversion of IAA flow toward the phloem, or an inhibition of lateral efflux from the sieve tubes. TIBA acts as an inhibitor of the auxin efflux carrier PIN1 (Morris et al., 2010). TIBA and other auxin transport inhibitors block the basipetal release of auxin by cells in the polar transport pathway, thereby causing auxin accumulation (Davies and Rubery, 1978). This auxin accumulation can result in lateral transport to neighboring cells or tissues (Nicolas et al., 2004), with the direction and rate of lateral transport determined by the prevailing auxin concentration gradient within the transport pathway. Such lateral auxin transport may be an integral...
component in auxin signaling pathways (Mattsson et al., 1999, 2003; Aloni, 2010). Cotyledons of Ricinus seedlings possess an extended bundle sheath that serves as a transport tissue and a temporal sink for assimilates (Rutten et al., 2003) and possibly also auxin.

Auxin transport inhibitors can alter the rate of lateral auxin efflux from cells. Mattsson et al. (1999) suggested that in developing Arabidopsis leaves the lateral movement of auxin toward the vascular strands was enhanced significantly by treatment with NPA (naphthylthalamic acid) or TIBA, as shown by the increased width of the developing veins. In transgenic Arabidopsis seedlings subjected to gravity or light stimulation, there was a tropic bending response of the hypocotyl which occurred concurrently with an elevated expression of the synthetic DR5::GUS auxin reporter gene on the more elongated side of the hypocotyl. The effect was attributed to the lateral relocation of the auxin efflux regulator PIN3 (Friml et al., 2002). Plants receiving gravity or light stimulation in the presence of NPA failed to show asymmetric DR5::GUS expression or tropic curvature, and NPA prevented the actin-dependent lateral redirection of auxin by inhibiting the relocation of the PIN3 protein in the cell plasma membrane.

It has been suggested that the action of auxin transport inhibitors is more broadly based through a general influence on cellular protein trafficking (Geldner et al., 2001), though Petrášek et al. (2003) consider that the inhibition of vesicle trafficking is not the mechanism by which phytotropins inhibit basipetal auxin transport, leaving the mechanism of phytotropin action unknown at the present time. In the present work, we show that in plants treated with 100 μM TIBA, [14C]sucrose transport is enhanced, as is [3H]IAA transport (Fig. 4B). Conceivably, the localization of sucrose transport is enhanced by a general inhibitory effect of TIBA on vesicle trafficking.
IAA transport during *Ricinus* germination

Factors including inorganic ions, pH, substrate concentration, as well as reagents for probing metabolic or transport activity (Komor, 1977; Maynard and Lucas, 1982; Williams et al., 1992; Schobert et al., 1998) can also be altered by TIBA as these proteins are degraded and turned over.

**Effect of potassium ion and sucrose concentration**

The uptake and phloem loading of sucrose are known to be controlled by a diverse set of internal or externally applied factors including inorganic ions, pH, substrate concentration, as well as reagents for probing metabolic or transport activity (Komor, 1977; Maynard and Lucas, 1982; Williams et al., 1992; Schobert et al., 1998). Given the complex role that sucrose and potassium ions seem to play in phloem function, we examined the effect of these factors on IAA transport. Phloem input and transport rates of IAA and of sucrose were

**Fig. 5.** Transport of \[\text{H}^3\text{IAA}\] (A) and \[\text{\}^{14}\text{C}\text{sucrose}\] (B) at various levels of sucrose. Each of the injection media contained 30 mM MES buffer at pH 6.3, 20 mM Na\(^+\), 0.71 MBq ml\(^{-1}\) \[\text{H}^3\text{IAA}\] (0.76 \(\mu\)M), and 0.63 MBq ml\(^{-1}\) \[\text{\}^{14}\text{C}\text{sucrose}\]. Non-radioactive sucrose was added to adjust concentration levels to 0.02, 20, and 100 mM. The data are combined from two experiments, and the means are from eight replicate measurements. For other conditions or comments see Fig. 2.

**Fig. 6.** Transport of \[\text{H}^3\text{IAA}\] (A) and \[\text{\}^{14}\text{C}\text{sucrose}\] (B) at various levels of sucrose in the presence of potassium ions. Each of the injection media contained 30 mM MES buffer at pH 6.3, 20 mM K\(^+\), 0.91 MBq ml\(^{-1}\) \[\text{H}^3\text{IAA}\] (0.98 \(\mu\)M), and 0.524 MBq ml\(^{-1}\) \[\text{\}^{14}\text{C}\text{sucrose}\]. Non-radioactive sucrose was added to adjust concentration levels to 0.02, 20, and 100 mM. The data are combined from two experiments, and the means are from eight replicate measurements. For other conditions or comments, see Fig. 5.
measured together at varying sucrose concentrations, with or without 20 mM K⁺ present in the IM; in the latter case, 20 mM Na⁺ was substituted for K⁺.

With the inclusion of 20 mM K⁺ in the IM, the pattern of sucrose transport was altered compared with that without K⁺. With 0.02 mM sucrose, the sucrose content of the exudate was ~0.9% at the end of the 1 h run (Fig. 6B), a value less than half of that obtained without K⁺ (Fig. 5B). Therefore, 20 mM K⁺ in the medium was inhibitory for sucrose transport, a finding in agreement with published results (Van Bel and Koops, 1985).

Also at this low applied sucrose level, the presence of potassium caused a shift in the time course from a largely linear to a strongly sigmoid shape, perhaps indicating a shift toward a longer loading pathway. With potassium present, there was no significant difference in the relative sucrose transport rates at the three applied sucrose levels, so that the sucrose flux increased in proportion to the applied concentration, suggesting that transport activity at the two higher levels was predominantly in its linear, non-saturable phase (Fig. 6B). Also, with higher applied sucrose levels, the value of \( t_{0.5} \) was much increased, indicating a lengthening loading pathway and a strong upward trend in the transient pool size (Fig. 6B); this may mean that a relatively greater portion of transported sucrose was passing through the mesophyll on its way to the phloem.

In the absence of potassium ions, the effect of sucrose concentration on the transport rates was either insignificant, as in the case of IAA (Fig. 5A), or inconsistent, as in the case of sucrose (Fig. 5B). The inclusion of 20 mM K⁺ in the IM evoked a set of correlated changes in IAA transport (Fig. 6A) that provide a striking contrast to the results obtained in the absence of K⁺ (Fig. 5A). At the lowest sucrose level, the amount of IAA nearly doubled after 1 h of transport due to the presence of potassium, presumably resulting from an enhancement of the plasma membrane H⁺ gradient with K⁺ acting as a counterion. Whereas the steady-state concentration of transported IAA in the phloem exudate in the presence of K⁺ was ~0.7% at 0.02 mM sucrose, it was reduced to ~0.3% and 0.15% at 20 mM and 100 mM sucrose, respectively (Fig. 6A). In addition, the \( t_{0.5} \) values in the presence of K⁺ declined from 23 min at 0.02 mM sucrose to 12 min and 6 min at 20 mM and 100 mM sucrose, respectively (Fig. 6A). These responses are in agreement with, and are explained by the combined effects of sucrose and K⁺ on phloem loading previously described. Therefore, the following conclusions may be drawn from the interactions of K⁺ and sucrose on IAA loading into the phloem (Figs 5A, 6A): (i) the stimulation of IAA loading by K⁺ suggests that the IAA carrier was in its high affinity phase at the applied concentrations of 20 mM K⁺ and 0.02 mM sucrose, and therefore the load-enhancing range of K⁺ for the IAA carrier must be wide enough to include the 20 mM level; (ii) the degree of sensitivity of IAA loading to the depolarization of the plasma membrane is correlated with sucrose concentration; and (iii) a \( t_{0.5} \) value may be taken as a semi-quantitative measure of the collective size of the intermediary pools within a given loading path. Because large pools would most probably be found outside the vascular tissues—the latter being of relatively limited volume—it is assumed that their probable location is in the mesophyll.

Our results regarding \( t_{0.5} \) values therefore suggest that at the lowest applied sucrose level IAA was being loaded primarily along a pathway passing through the protoplasts of mesophyll cells. At higher sucrose levels, the loading path was drastically diminished in size, suggesting that IAA loading was largely restricted to a direct transfer through the apoplast to the phloem, without passage through the mesophyll.

Inhibition of phloem transport by sulphydryl reagents

Photosynthates in leaves are generally loaded into the sieve element–companion cell complex through the plasma membrane from the apoplast or, alternatively, pass from the mesophyll to the phloem of minor veins through a symplastic pathway. Pathways may combine, run in parallel, or include a diffusive component depending on the species and on the physiological conditions within the tissue (Rennie and Turgeon, 2009). Evidence for the apoplastic loading of sugars and amino acids into the phloem has been provided for many plant species by testing their sensitivity to PCMBs, a membrane-impermeant inhibitor of proton-coupled transport (Lalonde et al., 2003). In Ricinus cotyledons, externally applied \(^{14}\)C)sucrose was shown to move to the sieve elements in two parallel pathways, directly from the apoplast and indirectly after transit through the mesophyll cells (Orlich and Komor, 1992). One of the Ricinus sucrose carriers expressed in yeast can be inhibited by PCMBs (Wei and Komor, 1996).

The transport of simultaneously applied \(^{3}H\)IAA and \(^{14}\)C)sucrose was measured with or without PCMBs or membrane-permeant p-chloromercuribenzoate (PCMB) to estimate the active, carrier-mediated component in their uptake. In the collected phloem exudate, a steady concentration level for both \(^{3}H\)IAA and \(^{14}\)C)sucrose was reached in all plants ~40–50 min after injection (Fig. 7). As judged by these equilibrium levels, the presence of PCMBs caused significant reductions in the active, carrier-based uptake of both \(^{3}H\)IAA and \(^{14}\)C)sucrose by ~25% and 40%, respectively (Fig. 7A, B). Marchant et al. (2002) have concluded that the auxin uptake carrier AUX1 facilitates IAA loading into the leaf vascular transport system, as an altered pattern of expression of an auxin-inducible reporter in Arabidopsis leaves was considered most consistent with impaired vascular loading. The observed responses suggest that in the loading pathway for IAA, the active component is relatively smaller than that for sucrose. Alternatively, the two carriers may differ in their sensitivity to the inhibitor. However, PCMBs also inhibits some aquaporins (Benga, 2003), which could upset water relations of the cells, so altering the observed responses.

With or without K⁺ present in the IM, PCMB inhibited IAA transport to, or nearly to, the same degree as did PCMBs (Fig. 7A, C; Na⁺ replaced K⁺ in Fig. 7C). In investigating uptake and movement of IAA in pea stems, Davies and Rubery (1978) found that whereas PCMBs decreased IAA accumulation in the stem segments, PCMB enhanced it. This was interpreted as penetrant PCMB blocking the IAA efflux carrier on the interior side of the lower plasma membrane, so retaining more IAA in the transporting cells. That export into Ricinus phloem was inhibited by both PCMB and PCMBs is, however, not surprising: as the cut phloem,
where transport was measured, involves an open-ended system, any build-up in the transporting cells due to carrier disruption would simply remain in those cells and never reach the phloem. Nonetheless in the absence of \(K^+\), PCMB was slightly less effective an inhibitor than PCMBS, matching the promotion of phloem accumulation by TIBA.

When \(K^+\) was excluded from the injection medium (with 20 mM \(Na^+\) substituted for \(K^+\) in the buffer), the inhibitory effect of PCMBS on IAA entry into the phloem was \(\sim54\%\) (Fig. 7C), more than twice the effect obtained with \(K^+\) present (Fig. 7A). Therefore, the presence of 20 mM \(K^+\) was inhibitory for the active component in IAA loading. Interestingly, potassium ions had the opposite effect on sucrose loading: in the absence of \(K^+\), PCMBS was wholly ineffective against sucrose transport (Fig. 7D). Perhaps in the latter case the active component of sucrose uptake was being disabled by the low proton motive force caused by the sharply reduced availability of \(K^+\) for charge compensation (Malek and Baker, 1978). However the active loading of IAA not only continued, but actually doubled in rate when potassium ions were withheld from the injection medium. This could be explained if the processes of IAA and sucrose loading are driven by metabolic energy derived from two distinct sources.

While PCMBS was only effective in reducing sucrose transport into the phloem with \(K^+\), PCMB was only effective in the absence of \(K^+\) (Fig. 7B, D; \(Na^+\) replaced \(K^+\) in Fig. 7D). The efficiency of each of the inhibitors may be differentially affected by the prevailing proton motive force that is expected to vary with the applied \(K^+\) concentration (see above). The observed effects of \(K^+\) on sucrose loading may involve the regulatory activity of \(K^+\) channels located in phloem cells together with \(H^+\) pumps and sucrose carriers. The loss of
AKT2/3 K+ channel function in an Arabidopsis mutant has been shown to result in impaired sucrose/H+ symporter activity and diminished phloem electric potential (Deeken et al., 2002).

Conclusion

In germinating Ricinus seedlings, both sucrose and IAA derived from the endosperm are transferred into the peri-cotyledory space and taken up by the cotyledons en route to the seedling axis (Komor, 1977). This would involve uptake by the cells of the cotyledons and then cell to cell transfer to the companion cells of the phloem. Alternatively, movement at some point may be apoplastic prior to transfer into the companion cells. Sucrose has been reported to use both these routes. The synthetic auxin NAA competitively inhibited the IAA accumulation in the phloem, showing that IAA was in part moving via auxin-specific transporters. PCMBS, which would act exterior to the cell membranes, reduced uptake of both IAA and sucrose by ~25% and 40%, respectively, indicating that carrier-mediated uptake into cells, not surprisingly, is involved at some point en route, and was more important for sucrose than for membrane-permeant IAA. As the IAA efflux carrier inhibitor TIBA enhanced IAA accumulation in the phloem, it would appear that the blocking of cell to cell IAA transport may force more IAA into the phloem, or that there is an efflux carrier in sieve tubes themselves preventing diversion to other cells en route. The presence of K+ at low sucrose concentrations doubled IAA loading into the phloem, whereas at 100 mM sucrose the loading of IAA was severely diminished in the presence of K+ even though sucrose without K+ had no effect. Thus the degree of sensitivity of IAA loading to the depolarization of the plasma membrane by K+ is correlated with sucrose concentration (the saturable influx via the proton co-transport system has a Km of ~25 mM in Ricinus cotyledons though the value for the outer layer is ~5 mM (Komor, 1977)). At the lowest applied sucrose level, IAA was being loaded primarily along a pathway passing through the protoplasts of mesophyll cells, but at higher sucrose levels IAA loading appeared to be restricted to a direct transfer through the apoplast to the phloem, without passage through the mesophyll. We conclude that the transport of IAA into the phloem is multifaceted, with a carrier-mediated pathway playing a significant role.

Acknowledgements

We thank the late Roger Spanswick, and Robert Turgeon and Philip Rubery for constructive comments.

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