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The Transport of Indole-3-Acetic Acid in Boron- and Calcium-Deficient Sunflower Hypocotyl Segments[1]

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ABSTRACT

Transfer of sunflower (*Helianthus annuus* L. cv Russian Mammoth) seedlings from complete nutrient solution to solutions deficient in either boron or calcium resulted in a steady decline in the rate of auxin transport, compared to seedlings that remained in the complete solution. In seedlings transferred to solutions deficient in both B and Ca, the decline in auxin transport was greater than seedlings deficient in only one element. The transfer of B- or Ca-deficient seedlings back to the complete solution prevented further decline in auxin transport, but auxin transport did not increase to the same level as seedlings maintained in complete solution. The significant reduction in auxin transport during the early stages of B or Ca deficiency was not related to (a) reduced growth rate of the hypocotyl, (b) increased acropetal movement of auxin, or (c) lack of respiratory substrates in the hypocotyl. In addition, no difference was found in the water-extractable total and ionic Ca in B-deficient and control nondeficient hypocotyls, indicating a direct effect of B on auxin transport, rather than indirectly by affecting Ca absorption. The rate of auxin transport in hypocotyls deficient in either B or Ca, was inversely correlated with K^+ leakage and rate of respiration. The data presented strongly support the view that there are separate sites for B and Ca in the basipetal transport of the plant hormone indoleacetic acid.

Recently it was demonstrated that Ca is essential in the basipetal transport or secretion of auxin in sunflower hypocotyl segments (5, 7). It was hypothesized that Ca probably functions in the same way that this element does in the secretion of many kinds of substances in animal cells (21). Basically, the model contends that the influx of Ca perturbs the cell, causing the secretion of the substance. It was thought that since the secretion of auxin occurs at the basal plasmalemma, then it is possible that this event could have been triggered by Ca that entered the basal end of the cell. If this contention of a basal entry of Ca is correct, and Ca pumps exist to maintain low cytoplasmic free Ca, we hypothesized that there could be an acropetal flux of Ca at the tissue or organ level. Investigation into this aspect revealed the existence of acropetal Ca efflux in 20 mm sunflower hypocotyl segments (3). Ca efflux was found to be enhanced by auxins, which in turn were rendered ineffective when applied together with the auxin transport inhibitor, 2,3,5-triiodobenzoic acid (3). Like basipetal auxin transport, the acropetal efflux of Ca was not affected by inverting the normal morphological orientation of the hypocotyl 180° relative to gravity. Also, the acropetal efflux of Ca was inhibited by cyanide and low temperature, just as the basipetal transport of auxin (4).

Although these findings support our hypothesis, it can be argued that our observations regarding the Ca-IAA transport relationship still appears to be more apparent than real. To probe this hypothesis further, we sought the use of other model systems. The main question is whether the findings of a Ca requirement in auxin transport, and the auxin promotion of Ca efflux at the hypocotyl level, is an accurate representation of the phenomenon at the cell level. It is possible that these findings are the direct effect of the overall disturbance in the hypocotyl, such as apical necrosis, increase membrane permeability, etc., resulting from Ca deficiency, and thus only indirectly to the deficiency of Ca. To this end we used the B-deficient seedling for the following reasons.

Both B and Ca are considered immobile elements in plant systems (19, 20). Deficiency or withdrawal of B or Ca from the root medium leads to necrosis of young tissues in roots and shoots, leaving the older portions of the plant relatively unaffected. The avid binding of Ca to the cell wall (23), and of H_3BO_3 to membrane and cell wall components with cis-hydroxyl configurations (14), together with the active extrusion of these two elements from the symplast (19, 20), are probably the main reasons for their relative immobility, or lack of retranslocation to other portions of the plant.

We presumed that if B-deficient sunflower hypocotyl segments also show an inhibition of auxin transport, then our hypothesis of a direct Ca-IAA transport relationship is probably incorrect, and that the results we observed were only indirectly related to Ca deficiency. Results presented in this communication will show that auxin transport was also inhibited in B-deficient hypocotyl. However, data will also be presented showing that the reason for the inhibition of auxin transport is not as presumed above, but that B-dependent sites, independent of the site(s) for Ca, are also present in auxin transport.

MATERIALS AND METHODS

Plant Materials. Sunflower (*Helianthus annus* L. cv Russian Mammoth; Olds Seed Co., Madision, WI) seeds were germinated between paper towels moistened with distilled H_2O. After about 1 d, the seeds with good radicle growth were selected and transferred to one-fourth strength Hoagland solution A (12) containing 23 μM H_3BO_3 (0.25 μg/ml B) in 500 ml plastic bowls with fiberglass screening stretched on top to support the seedlings. The seedlings were subjected to 16 h photoperiod (mixed fluorescent and incandescent lamps; net intensity of 13.5 $J \cdot m^{-2} \cdot s^{-1}$) and 27 to 30°C when the lights were on, 24 to 26°C when off. After 3 d in the complete solution, the roots were thoroughly rinsed in distilled H_2O and the seedlings transferred to the following solutions for treatments: (a) nondeficient, same solution as indicated above; (b) B-deficient, same solution; H_3BO_3

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not added; (c) Ca-deficient, same solution; $Ca(NO_3)_2$ replaced with $NaNO_3$; (d) B and Ca-deficient, as in (c), and H_3BO_3 was not added. Further transfers to other growing solutions are described in the figure legends.

Measurement of Auxin Transport. With the use of a cutter with multiple fixed blades spaced 5 mm apart, three segments were cut from each hypocotyl beginning about 5 mm below the cotyledonary node. Twelve segments were placed apical end down on donor agar discs (1% agar; 1.1 cm diameter, about 0.1 ml) containing 10 μM [1-^{14}C]IAA (13.5 mCi/mM). A receiver agar disc was placed in contact with the basal end for 4 h. To determine the acropetal movement of auxin, the receiver agar disc was placed at the apical end of the hypocotyl segment and the donor agar at the basal end. Each treatment was replicated three times. The transport setup was placed inside a moist chamber in the dark. After the transport period, the receiver agar disc was transferred to a piece of filter paper, placed inside a scintillation vial and dried at 80°C. The standard toluene-based scintillation counting solution (with POPOP and PPO) was added after drying the sample. Radioactivity was determined using a Packard Tricarb Spectrometer.

Measurement of Potassium Leakage. Twenty-four 5 mm long hypocotyl segments from eight seedlings were placed in a 120 ml plastic beaker with 10 ml 0.1 mM KCl. The beakers were placed on a shaker at 90 to 100 strokes/min. At the end of the incubation period, the segments were removed and the K^+ content of the medium assayed using a K^+-specific electrode (Orion 93-19) together with a single junction reference electrode (Orion 90-01) attached to an Orion 901 research microprocessr ionalyzer. Data presented is net K^+ increse in the incubation solution.

Measurement of Respiration. Respiration was measured with a Yellow Springs Instruments Co. model 53 O_2 monitor equipped with a Clark-type electrode. Ten 1 cm long hypocotyl segments were cut beginning about 5 mm below the cotyledonary node and placed in a small cheesecloth bag, which was placed inside the glass chamber with 10 ml 10 mM Tris-Mes buffer (pH 6.5) and stirred with a magnetic bar. The electrode was calibrated with air-saturated buffer solution before use. The temperature was maintained at 27°C with a circulating water bath. A potentiometric recorder attached to the O_2 meter indicated the decrease in dissolved O_2 in the chamber.

Measurement of Water-Extractable Calcium. Thirty-six 5 mm long hypocotyl segments from 12 seedlings were wrapped in two layers of Miracloth moistened with deionized distilled H_2O, and subjected to a pressure of 10,000 pounds load for 30 s in a Carver Laboratory Press. The press cake was washed twice with deionized distilled H_2O and subjected to the same pressure. The extracts were combined and the total volume made up to 10 ml with distilled H_2O. Ionic Ca was determined in this extract using a Ca^{2+}-specific electrode (Orion 93-20) together with a single junction reference electrode (Orion 90-01). After determination of the ionic Ca, the solution was transferred to a crucible, dried overnight, and then placed in a muffle furnace at 600°C for 16 h. The ash was dissolved in 9 ml 0.1 N HCl and the pH adjusted to 5.5 to 6.0 with KOH. The final volume was made to 10 ml with distilled H_2O. The Ca^{2+}-specific electrode was used to determine the Ca in solution and considered here as the total water-extractable Ca.

RESULTS

Seedling Growth. One of the first signs of abnormal development in the seedlings was observed about a day after transfer of the 4 d old seedlings to the B-deficient solution. The length of the main roots was noticeably shorter than the roots of seedlings that remained in the complete solution. Subsequently, lateral roots started to appear but their elongation was also inhibited.

Transfer of the seedlings to the Ca-deficient solution caused the roots to become translucent after 1.5 to 2 d. Subsequently, the whole root system turned yellow-brown. Compared to the roots that remained in the complete solution, the Ca-deficient roots showed inhibition of growth and development, although the roots did not look as abnormal as the B-deficient roots.

The leaves just starting to form at the time of transfer of the seedlings to the B-deficient solution developed an abnormal shape and bronze coloration. The leaves that succeeded were either necrotic, wrinkled, brittle, or narrow. Chlorosis, smaller size or distorted leaves observed in the seedlings, 4 to 5 d after transfer to the Ca-deficient solution.

The inital length of the hypocotyl at d 4, after 3 d in complete solution, was 6.4 cm. Transfer of the seedlings to a new batch of complete solution resulted in a net growth of 4.5 cm at 8 d, while transfer of the seedlings to B- or Ca-deficient solution caused a net growth of 3.9 cm. Only in the case of seedlings subjected for 4 d to both B and Ca deficiencies was total growth significantly reduced (25). Most of the auxin transport measurements were performed using seedlings that had been in the deficient solution for 2 d.

Effect of Boron and Calcium Deficiencies in Auxin Transport. The seedlings were in complete one-fourth strength Hoagland solution for 3 d following 1 d of germination. The treatments were started on the 5th d by transfer of the seedlings to new one-fourth strength Hoagland solution, or solutions deficient in either B or Ca, or both. The effect of these treatments on auxin transport is shown in Figure 1. Auxin transport in seedlings that remained in complete solution decreased only slightly at 8 d compared to the start of the treatments at 5 d. Significant increases in auxin transport were observed 24 to 48 h after transfer of the seedlings to the new complete solution.

Seedlings transferred to the B- or Ca-deficient solution showed a gradual decline in auxin transport. The data in Figure 1 show significant reduction in auxin transport 48 h after transfer of the seedlings to the deficient media; most of the latter experiments showed small but significant reduction in auxin transport as early as 24 h after transfer of the seedlings. Hypocotyls deficient in both B and Ca showed the earliest and highest rate of reduction in auxin transport.

The decrease in basipetal auxin transport is not due to a loss of polarity of transport. The deficiency of either B or Ca did not affect the rate of acropetal movement of auxin, even in the case where basipetal auxin transport was significantly inhibited (Fig. 2).

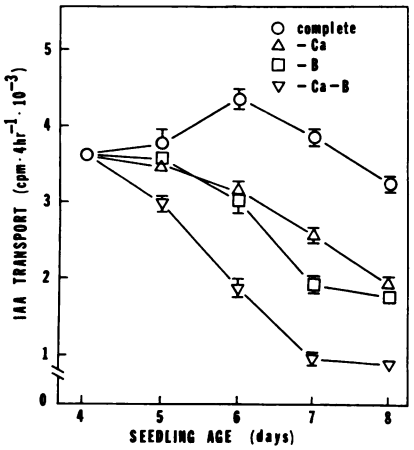

FIG. 1. Effect of deficiency and seedling age on auxin transport. All the seedlings were in complete solution until transferred to solutions deficient in B or Ca for 2 d before they were used on the 6th d. The basipetal transport of [1-^{14}C]IAA was measured using 12, 5 mm hypocotyl segments replicated three times. SE is indicated by the vertical bars when it exceeds the size of the symbol.

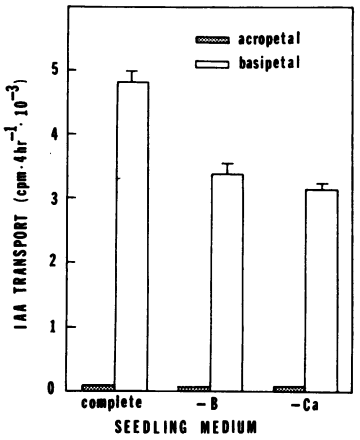

FIG. 2. Acropetal and basipetal transport of [1-^{14}C]IAA in hypocotyl segments of 6 d old seedlings. The seedlings were in complete nutrient solution except for the last 2 d when transferred to B-, or Ca-deficient solution. Each value represents the mean of three replications each with 12, 5 mm hypocotyl segments. The vertical bars represent the SE.

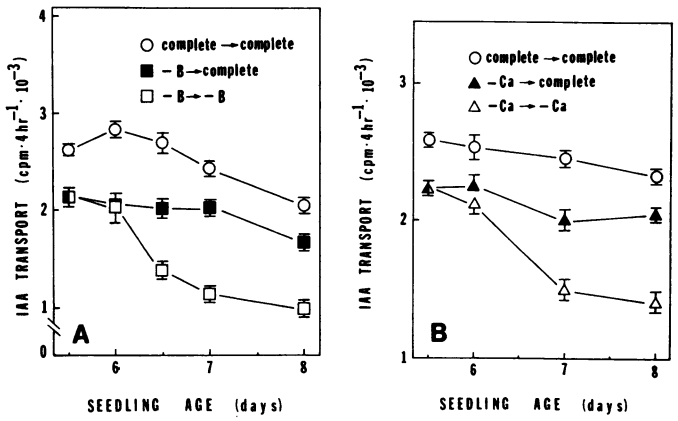

FIG. 3. Recovery of auxin transport on transfer of deficient seedlings to complete solution. After 3 d in complete solution, the seedlings were transferred to B- or Ca-deficient solution for 36 h. The 5.5 d old seedlings were then transferred back to complete solution, or to the original deficient solution. Auxin transport was measured as described in the text.

Transfer of Deficient Seedlings Back to Complete Solution. Auxin transport was significantly inhibited after 36 h in either a B- of Ca-deficient solution (Fig. 3). Transfer of these seedlings back to the complete solution prevented the further decline in auxin transport but did not result in an increase to the same level as that of the control plants that had always been in the complete solution.

Respiration and Potassium Leakage. Figure 4 shows respiration and K^+ leakage in hypocotyl segments 24 h after transfer of the seedlings to solutions deficient in either B, or Ca, or both. The overall results showed a positive correlation between O_2 absorption and K^+ leakage in the hypocotyl. Control segments with a normal supply of B and Ca showed the lowest rate of respiration and K^+ leakage; deficiency of either or both elements resulted in an increase in K^+ leakage and respiration.

Effect of Boron on Water-Extractable Calcium. One early suggested role for B was that it increased the concentration of Ca in the expressed sap or water extract of plants (16). If true, this would mean that Ca absorption, or Ca participation in physiological reactions would be impaired in plants subjected to B deficiency. Thus, there is the possibility that auxin transport inhibition in B-deficient hypocotyl may actually be due to a deficiency of Ca in the tissues, although enough Ca is present in the root solution.

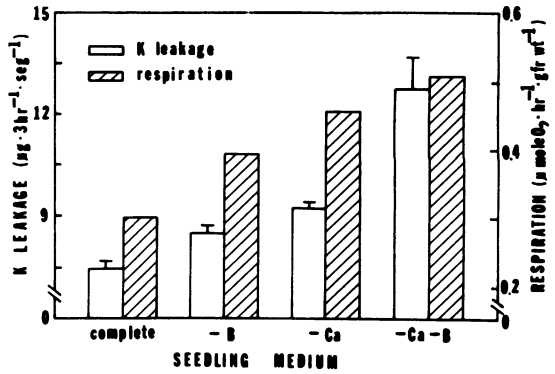

FIG. 4. Effect of deficiency on potassium leakage and respiration. Potassium leakage was determined as the net amount of K^+ that leaches out from 24, 5 mm hypocotyl segments in 10 ml 0.1 mM KCl. A Clark-type O_2 electrode was used to monitor the decrease in dissolved O_2 in the incubation medium.

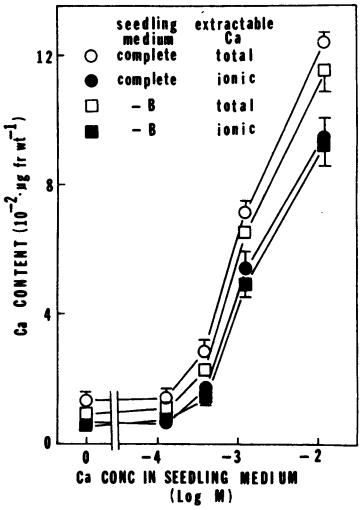

FIG. 5. The effect of B deficiency on the concentration of water-extractable Ca. The seedlings were in complete solution until transferred to solutions with increasing concentrations of Ca for 2 d, in the presence or absence of added H_3BO_3. The seedlings were used on the 6th d. The hypocotyls were extracted with a small amount of distilled H_2O in a Carver Press. Each value represents the mean of four replications, each with 36, 5 mm hypocotyl segments. A Ca^{2+}-specific electrode was used to determine the Ca in the extract before (ionic) and after ashing the extract (total Ca). The vertical bars represent the SE.

The above hypothesis was tested by determining the total and ionic Ca in the hypocotyl extracts of seedlings subjected to B deficiency for 2 d. Figure 5 shows an increase in total and ionic Ca in the hypocotyl extracts as the Ca in the root solution was increased. However, the presence or absence of B in the solution did not result in a significant change in either total or ionic Ca in the hypocotyl extracts. Therefore, the inhibition of auxin transport in the B-deficient hypocotyl cannot be due to the purported role of B in affecting Ca availability.

Incubation of Hypocotyl Segments in Sucrose Solution. Another postulated function for B is that this element is essential for the translocation of carbohydrates in plants (10). If true, this could mean that the reduced rate of auxin transport in B-deficient hypocotyls could be due to a deficiency of respiratory substrates brought about by an impairment of its translocation from the cotyledons. Attempts were made to supplement the possible lack of respiratory substrates by incubating the segments in 2% su-

crose solution prior to the measurement of auxin transport.

The only change brought about by the incubation in sucrose solution, whether or not H_3BO_3 was present, was a tendency to inhibit auxin transport (Fig. 6). Incubation in B solution caused a slight but significant increase in auxin transport, compared to segments incubated in water. More data on this question is shown in the accompanying manuscript (26).

DISCUSSION

Evidence for the Presence of Boron and Calcium Sites Involved in Auxin Transport. The present results confirm the earlier findings that Ca is essential in the basipetal transport of auxin (5, 7). In addition, the results show that a deficiency of the equally immobile element boron can likewise lead to the inhibition of auxin transport (Fig. 1).

The inhibition of auxin transport in the B-deficient hypocotyl could not have been due to a deficiency of Ca, since the concentration of Ca in the root medium of the B-deficient seedling was the same as in the control complete solution. Furthermore, transfer of plants with incipient B deficiency to new solutions with Ca but without B, only led to further decline in auxin transport. However, the transfer of identically treated plants to solutions with B caused transport to stabilize as in the control, nondeficient plants (Fig. 3A). Likewise, auxin transport in the Ca-deficient hypocotyl can be prevented from further decline only upon transfer of the seedlings to solutions with Ca and B, not in solutions with B and without Ca (Fig. 3B).

The existence of specific sites for B and Ca in auxin transport is also suggested by the faster rate of decline in plants deficient in both elements, compared to those deficient in only B or only Ca (Fig. 1).

The interaction between the two elements, particularly the possible effect of B on available Ca (16), can also be discounted. B-deficient hypocotyls that showed inhibition of auxin transport, were found to have the same content of total and water-extractable Ca as the control nondeficient hypocotyl (Fig. 5).

Effect of Polarity, Sucrose, and Hypocotyl Growth on Auxin Transport. The transport of auxin in the sunflower hypocotyl tissue is highly polar, being essentially basipetal (6). Since there is only a finite amount of IAA in the cell, it is logical to suppose that a treatment causing the promotion of acropetal auxin movement would lead to a decrease in basipetal auxin transport. However, neither B nor Ca deficiency caused a change in acropetal auxin movement, despite the significant reduction of basipetal auxin transport compared to the control nondeficient hypocotyls (Fig. 2). These data suggest that, barring changes in transmembrane H^+ gradient that could affect the ratio $IAAH/IAA^-$, the primary control of auxin transport in individual cells is at the site of auxin secretion at the basal plasmalemma.

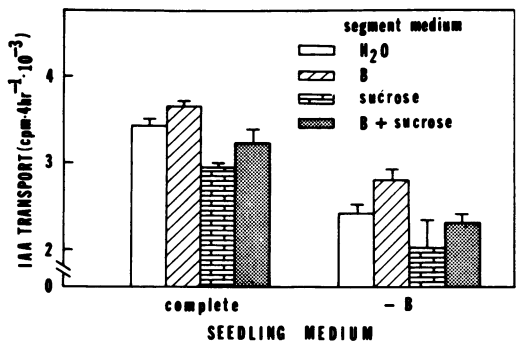

FIG. 6. Effect of B and sucrose on auxin transport. Hypocotyl segments from 6 d old seedlings in complete solution throughout, or in B-deficient solution for the last 2 d, were incubated in 2% sucrose and/or 0.46 mM H_3BO_3 for 2 h before measurement of auxin transport. Each value represents the mean of three replicates each with 12, 5 mm hypocotyl segments. The vertical bars represent SE.

Boron has been hypothesized to be essential in the translocation of carbohydrates in plants (10). Thus, there is the possibility that the reduction in auxin transport in B-deficient hypocotyl could be due to a deficiency of respiratory substrates. However, the incubation of hypocotyls in 2% sucrose, a concentration normally used in bioassay systems, tended to inhibit rather than promote auxin transport, suggesting the possibility that respiration substrates may not be limiting (Fig. 6).

It is also important to note that significant reduction in auxin transport could be detected 24 to 36 h after transfer of the seedlings to the deficient solutions. At this time no significant reduction in growth rate of the hypocotyl was detected (25).

Membrane Permeability and Hypocotyl Segment Respiration. The role of Ca in membrane permeability and selectivity in plant and animal cells is well known (8, 15). The ionic groups on the polar head of acidic phospholipids in membranes bind Ca and other ions (2). The presence of Ca on the membrane causes gelling and greater hydrophobicity leading to membrane rigidity.

Boron which is not essential to animals, is also thought to have a major role in the proper functioning of plant membranes (11, 17, 24). Boron binds to organic molecules with cis-hydroxyl configurations as in some phenols, sugars, and its derivatives (14). The possible binding of B to sugars of the glycolipids and glycoproteins on the surface is probably the most significant in the present context.

The high rate of K^+ leakage in the Ca-deficient hypocotyl (Fig. 4) confirms the observations of many that an inverse relationship exists between Ca concentration in the medium and leakage of cell contents (1, 18, 28). Similarly, a high rate of K^+ leakage was found in B-deficient hypocotyls (Fig. 4), although this has not been confirmed in other species. In B-deficient cells of the diatom, *Cylindrotheca fusiformis*, the influx of ^{86}Rb is less compared to nondeficient cells. However, the rate of efflux is also less compared with normal cells, thus leading to a higher content of the radionuclide in the B-deficient cells (22). A reduced rate of chloride and phosphate influx was noted in B-deficient maize roots, compared with nondeficient roots; however, efflux was found to be unaffected by the B status (17). Hirsch and Torrey (11) found an increase in cell wall thickness and loss of membrane integrity as the primary result of B deficient roots of sunflower. Kouchi and Kumazawa (13), on the other hand, did not associate the increase in cell wall thickness in cells of B-deficient tomato roots with the degradation of the membrane systems. These discrepant observations may be related to species differences, or simply to the degree or stage of deficiency. In any case, these obesrvations seem to suggest that B is probably more specifically involved with ion carriers or channels that affect ion fluxes, while Ca is more generally involved with the packing of the phospholipids, and thus general leakiness of the membrane.

Figure 4 also shows that the high rate of K^+ leakage in both B- and Ca-deficient hypocotyls is accompanied by a higher rate of respiration relative to normal hypocotyls. An inverse relationship is known to exist between tissue Ca content and respiration in apple fruit slices (1, 9). The high rate of respiration in low-Ca apple fruits was attributed to leakage of respiratory substrates from the vacuole to the cytoplasm (1). A higher rate of respiration was also observed in *C. fusiformis* cells deficient in B, compared with normal cells (22). Timashov (27) attributes the high rate of respiration in B-deficient sunflower leaves to an uncoupling of the electron transport pathway from ATP synthesis.

In summary, both B-deficient and Ca-deficient sunflower hypocotyl segments showed similar responses of increased rate of K^+ leakage, high rate of O_2 absorption, and a reduced rate of basipetal auxin transport, compared with nondeficient hypocotyl. At this point the specific number of sites involved in auxin

transport affected by deficiency of each element is not clear. In the accompanying manuscript (26) we detail how we distinguished and characterized two possible sites each for B and Ca involved in auxin transport.

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