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Distribution of Indole-3-Acetic Acid in the Apoplast and Symplast of Squash (*Cucurbita maxima*) Hypocotyls

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The concentration of endogenous IAA was higher in an apoplastic solution (2.3×10^{-7} M) than in a symplastic solution (0.5×10^{-7} M) obtained from segments of etiolated squash (*Cucurbita maxima* Duch.) hypocotyls. Exogenously applied IAA (10^{-5} M) increased the level of IAA in both the apoplastic and the symplastic solution. The concentration of IAA in the apoplastic solution increased to 75% of the concentration of exogenous IAA in 4 h, but that in the symplastic solution increased only to 23% of the concentration of exogenous IAA. These results demonstrate that the concentration of endogenous IAA is higher in the apoplast than in the symplast of squash hypocotyls, and they suggest that IAA exerts its physiological effects, at least to some extent, from the outside of cells.

Key words: Apoplast — *Cucurbita maxima* — Hypocotyl — IAA — Squash — Symplast.

IAA is known as a natural plant hormone that regulates plant growth. IAA induces the rapid elongation of cells in isolated sections of stems and coleoptiles. The response begins within 10 min after the addition of IAA and results in a 5- to 10-fold increase in the growth rate. Moreover, the effect persists for hours or even days (Evans 1985).

Several researchers have claimed that, to exert its effects, IAA must bind first to a receptor. This receptor could be on the outside of the plasma membrane or at some internal site within the cell (Rayle and Cleland 1970) but it is certainly not in the wall itself (Jones 1990). Putative receptors were reported to be of two types, namely, an auxin-binding protein at the plasma membrane and a protein that is solubilized in the cytoplasm. Löbler and Klämbt (1985) reported that an auxin-binding protein, located at the plasma membrane, recognized auxin and was concentrated in the outer epidermis, as compared to the inner tissues of maize coleoptiles. Venis et al. (1990) demonstrated that exogenous application of impermeant analogs of auxin caused a rapid response (hyperpolarization of the plasma membrane) in tobacco mesophyll protoplasts, as well as long-term elongation growth of segments of pea epicotyls. The results suggest that auxin causes a rapid growth, related response in plant cells by exerting its effect on cells from the outside of the cell and, moreover, that auxin does not necessarily need to enter the cell to exert its effect on elongation growth.

Optimal concentrations of exogenous IAA for the elongation of sections of stems and coleoptiles are usually in the range of 10^{-5} to 10^{-6} M (Nissen 1985), but measured concentrations of endogenous IAA extracted from sections of growing zones of squash hypocotyls were only about 4×10^{-8} M (Sakurai et al. 1985). The reason for the difference between the optimum concentration of exogenously applied IAA and the endogenous concentration has not yet been elucidated. There is a possibility that the low concentration of endogenous IAA found in the tissue is due to the low concentration in the symplast. The volume of the symplast is more than ten times that of the apoplast. Even if endogenous IAA were to be distributed preferentially in the apoplast, the concentration of IAA extracted from whole tissues could be estimated to be lower than the concentration in the apoplast.

To demonstrate directly that IAA is distributed preferentially in the apoplast and to determine whether it is likely that IAA acts from the outside of cells, we measured levels of IAA in the apoplast and symplast of growing tissues of etiolated squash hypocotyls.

Materials and Methods

Plant materials—Squash seeds (*Cucurbita maxima* Duch. cv. Houkou-Aokawaamaguri; Takayama Seed Co., Kyoto) were soaked for 16 h in tap water and then they were allowed to germinate for 2 d in darkness on two layers of moistened filter paper. The germinated seeds were placed on a stainless-steel mesh in plastic boxes (34 cm × 23.5 cm × 4.6 cm) that contained two liters of 1/5-strength Hoagland solution, as described previously (Sakurai et al. 1985), and they were cultured hydroponically at 25.5±0.5°C for 2 or 3 d in darkness. After 2 or 3 d, seedlings that had hypocotyls of 4–6 and 8–10 cm in length, respectively, were selected for experiments.

Collection of apoplastic and symplastic solutions—Apoplastic solution was collected by the centrifugation method described previously by Sakurai and Kuraishi (1988). Hypocotyl segments (1 cm in length) were excised from the upper part of hypocotyls, 5 mm below the cotyledonary node, and from the lower part of hypocotyls, 5 mm above the hypocotyl base. The segments were

Abbreviations: AP, apoplast; GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; SY, symplast.

placed on a stainless-steel mesh in the barrel of a 6-ml plastic syringe (13 mm i.d.) which had been cut off at the 5-ml mark and was placed on an Eppendorf tube (1.5 ml) in a centrifuge tube (22.5 ml).

The tube was centrifuged for 20 min at 4,500×*g* at 4°C for collection of apoplastic solution (AP solution) from the segments. The segments were frozen in liquid nitrogen immediately after centrifugation, thawed and recentrifuged for 20 min at 4,500×*g* at 4°C for collection of cell sap (symplastic solution; SY solution). Weights of AP and SY solutions were calculated by subtracting the weight of the Eppendorf tube before collection of the AP or SY solution from the weight of the Eppendorf tube after collection of the AP or SY solution.

Extraction of IAA from the apoplastic and symplastic solutions—The AP and SY solutions in Eppendorf tubes were adjusted to pH 2.8 by two methods. In one method, solutions in five tubes were each adjusted to pH 2.8 with 200 μl of 2 M citric acid–NaOH buffer, and then the solutions were combined in a single test tube. In the other method, solutions of each of five tube were transferred to one test tube, then an aliquot of 2 M H_3PO_4, the volume of which was 10% the volume of the pooled solution, was added to the solution in the test tube. The two methods gave similar results.

[$^{13}C_6$]IAA (2 nmol; Merck, Sharp, and Dohme, Canada Ldt.) was added to the sample solution in the test tube as an internal standard. The solution was then extracted twice with diethyl ether. The organic phase was evaporated to dryness under N_2 gas at room temperature or in a rotary evaporator (the recovery range from 90 to 100%). We also attempted to evaporate the organic phase to dryness with a stream of filtered air, but the recovery of IAA in such cases was fairly low (less than 10%). Therefore, this method was not used in these experiments. Each dried sample was dissolved in 0.9–1.5 ml of 25% acetonitrile that contained 20 mM acetic acid.

HPLC—IAA was purified by the method described by Akiyama et al. (1983) on an Inertsil ODS-2 column (4.6 mm i.d.× 150 mm; GL Sciences Inc., Tokyo) or on a Puresil C_{18} 120 Å column (4.6 mm i.d.×150 mm; Nihon Millipore Ltd, Tokyo). Columns were eluted isocratically with 25% acetonitrile that contained 20 mM acetic acid at a flow rate of 0.8 ml min^{-1}. The HPLC system consisted of a pump unit (model LC-6A; Shimadzu Inc., Kyoto) connected to a system controller (model SCL-6A; Shimadzu Inc.). IAA was monitored with a fluorometric detector (model FP-200; Japan Spectroscopic Co., Tokyo) with excitation at 280±5 nm and emission at 350±5 nm. The IAA-containing fractions were collected for analysis by GC-SIM-MS.

GC-SIM-MS—After HPLC, appropriate IAA-containing fractions were pooled and evaporated to dryness on a rotary evaporator. Samples were dissolved in methanol and transferred to test tubes (the recovery range from 90 to 100%). We also attempted to dissolve dried samples in diethyl ether but the recovery of IAA was very low (less than 10%). Therefore, we did not use diethyl ether for dissolving the dried samples. Each sample in a test tube was evaporated to dryness under N_2 gas. The dried sample in the test tube was methylated with diazomethane, which was generated with a diazomethane generator (Wheaton, Millville, NJ, U.S.A.). IAA in the methylated sample was quantitated by GC-SIM-MS (model QP-1000; Shimadzu Inc.). Ionization of the compound was achieved by electron impact at 70 eV. The gas chromatograph was equipped with a 30-m fused silica capillary column (CBJ 17; Shimadzu Inc.). IAA content was calculated from the ratio of the peak area of [$^{13}C_6$]IAA (m/z=136) to that of the natural IAA (m/z=130).

Treatment of segments of squash hypocotyls with IAA—One-cm-long segments were incubated at 25.5±0.5°C in darkness in a Petri dish that contained 10 ml of water with or without 10^{-5} M IAA. All manipulations were conducted under dim green light. After incubation, the segments were blotted with a Kim-wipe and placed vertically in a plastic syringe for collection of AP and SY solutions as described above. To estimate the amount of IAA that was transiently associated with the surface of segments but had not been absorbed by the segments, we dipped the segments in a solution of IAA for one second, then blotted and treated them as above. To calculate the exact amount of IAA in AP and SY solutions, we used the following equation:

$$\text{Amount of IAA} = A - (B - C)$$

where A is the amount of IAA in the AP or SY solution from IAA-treated segments after a given incubation period; B is the amount of IAA in the AP or SY solution from segments treated with IAA for one second; and C is the amount of IAA in the AP or SY solution from untreated segments.

Results

Apoplastic and symplastic endogenous IAA in up-

Table 1 Levels of endogenous IAA in the apoplast and symplast of upper and lower segments of squash hypocotyls

Day	Part of hypocotyl	Amount of IAA (pmol $segment^{-1}$)		Weight (mg $segment^{-1}$)		Concentration of IAA (10^{-8} M)	
		AP (a)	SY (b)	AP (A)	SY (B)	AP (a/A)	SY (b/B)
2	Upper	0.44	2.86	1.08	53.16	40.54	5.38
	Lower	0.37	2.05	8.75	75.65	4.17	2.71
3	Upper	0.47	2.57	4.79	66.12	9.71	3.89
	Lower	0.29	1.39	16.55	79.87	1.76	1.74

One-cm-long segments were excised from upper (0 to 1 cm from the hook) and lower (0 to 1 cm from the base) parts of etiolated hypocotyls of squash seedlings after growth for 2 and 3 d. AP and SY solutions were collected by centrifugation. Levels of endogenous IAA in AP and SY solutions were determined by GC-SIM-MS. The concentration of IAA was calculated by dividing the amount of IAA by the weight (in grams) of AP or SY solution.

Table 2 Levels of endogenous IAA in the apoplast and symplast of the upper segments of hypocotyls from 2-d-old seedlings

	Amount of IAA (pmol segment^{-1})	Weight (mg segment^{-1})	Concentration of IAA (10^{-8} M)
AP	0.37±0.02[a]	1.64±0.15	22.58±2.79
SY	2.67±0.16	54.52±0.52	4.90±0.33

One-cm-long segments were excised from the upper, growing zone (0 to 1 cm from the hook) of etiolated hypocotyls of squash seedlings that had been grown for 2 d in darkness.
[a] Data are means±SE (n=3).

per and lower segments of hypocotyls—Table 1 shows amounts and concentrations of endogenous IAA in AP and SY solutions obtained from upper and lower segments of hypocotyls of 2- or 3-d-old squash seedlings. The absolute amount of IAA in AP solutions was always lower than that in SY solutions for both upper and lower segments of hypocotyls of both 2- and 3-d-old seedlings. The upper segments yielded slightly larger amounts of IAA in the AP and SY solutions than the lower segments.

The concentrations of IAA were calculated from the weights of AP and SY solutions, on the assumption that the weight of each solution in grams was close to its volume in milliliters. Calculated concentrations of IAA in AP solutions from the upper segments were 8 times those in SY solutions on day 2. On day 3, the difference was smaller but was still 2.5-fold. Not surprisingly, the concentration of IAA in AP and SY solutions was always higher in the upper, growing region, than in the lower, non-growing region, on days 2 and 3. In the lower region, the difference between concentrations of IAA in AP and SY solutions was negligible on day 3.

Endogenous IAA in upper segments of hypocotyls from 2-d-old seedling—We confirmed the level of IAA in upper hypocotyl segments of 2-d-old seedlings (Table 2). The data in Table 2 are the averages of results from three independent experiments. The weight of the AP solution was approximately 3% of that of the SY solution. The amount of IAA in the SY solution was 7.2 times that in the AP solution, while the calculated concentration of IAA in the AP solution was 4.6 times that in the SY solution. The results confirmed that the concentration of IAA in the AP solution of the growing zone of the hypocotyl was higher than that in the SY solution.

Amounts of IAA in IAA-treated segments—When segments are treated with exogenous IAA, the concentration of IAA in the AP solution should be always higher than that in the SY solution. Furthermore, such results would validate our method for collecting AP solution. Figure 1A shows changes in amounts of IAA in AP and SY solutions in IAA-treated segments. The amount of IAA in both AP and SY solutions in the segments increased up to 4 h. Calculated concentrations of IAA in AP solutions were always higher than in SY solutions. The concentration of IAA in the AP solution reached 75% of the concentration of exoge-

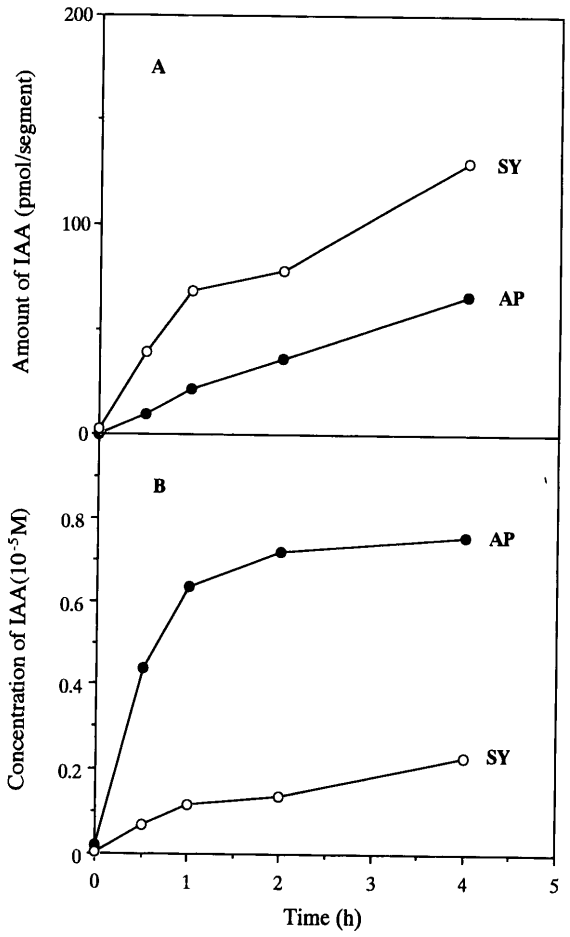

Fig. 1 Time course of changes in the amount (A) and in the concentration (B) of IAA in AP (●) and SY (○) solutions from hypocotyl segments that had been treated with 10^{-5} M IAA. Segments of squash hypocotyls (1 cm in length) from 2-d-old seedlings were incubated with 10^{-5} M IAA, then AP and SY solutions were separately collected by centrifugation. The amounts of IAA (A) in AP and SY solutions were determined by GC-SIM-MS. The amount of IAA was divided by the weight (in grams) of the AP or SY solution to calculate the concentration of IAA (B).

nous IAA (10^{-5} M) and that in the SY solution reached only 23% in 4 h (Fig. 1B). The difference in concentrations of IAA between AP and SY solutions decreased from 6.4-fold at 0.5 h to 3.3-fold after 4 h of treatment with IAA.

The concentration of IAA in the AP solution from segments that had been incubated in water for 2 h was below the limit of detection (below 6.20×10^{-8} M), but the concentration of IAA in the SY solution from segments that had been incubated in water for 2 h was still 4.46×10^{-8} M, being similar the concentration of endogenous IAA in the SY solution from intact segments, namely, 4.90×10^{-8} M (Table 2).

Discussion

In the present experiments, we used a centrifugation method to collect AP and SY solutions. The relative weight of AP solutions as a percentage of the total weight of solutions collected by centrifugation ranged from 2 to 17% (Table 1). Values were higher for the lower part than for the upper part of the hypocotyl. The values on day 2 for the upper and the lower part of the hypocotyl were lower than those on day 3. The volume of AP solution in growing stem tissues of various plants, as a percentage of tissue volume, was estimated to be about 4% in young growing epicotyls of pea by a totally different method (Cosgrove and Cleland 1983). Sakurai and Kuraishi (1988) reported that the volume of the AP solution in the upper part of the squash hypocotyl was always lower than in the lower part, and the volume of the AP solution in young hypocotyls was always lower than that in old hypocotyls. These results support the validity of our centrifugation method for collection of AP solution.

We identified and quantitated IAA in the AP solution by GC-SIM-MS and a centrifugation method. The presence of endogenous IAA in the AP solution was demonstrated for the first time in this study. The concentration of endogenous IAA was always higher in the AP than in the SY solution from squash hypocotyls, although the concentration in the AP solution (2.3×10^{-7} M) was lower than the optimal concentration (10^{-5} M) for induction of elongation growth by exogenously applied IAA. The concentration of exogenous IAA that induces a directly related response in terms of the extent of elongation ranges from 1 to 6×10^{-7} M (Nissen 1985). Within this range, plants respond to the hormone very effectively: a small change in the concentration of IAA is sufficient to alter the growth rate. By contrast, around the optimum concentration of IAA, a small change in the concentration has only a small effect on the growth rate. The concentration of endogenous IAA in the AP solution (2.3×10^{-7} M) determined in this study falls within the range that is directly related to the extent of the response. These results support the hypothesis that IAA exerts its physiological effect on the cell from outside of the plasma membrane (Löbler and Klämbt 1985, Venis et al. 1990).

The concentration of IAA in the AP solution from the upper part was higher on day 2 than on day 3. The upper part corresponds to the growing zone in the squash hypocotyl. It elongates at the maximum rate on day 2 and elongation slows down on day 3 (Sakurai et al. 1985, 1987). The decrease in the concentration of IAA in the AP solution from the upper part on day 3 corresponded to the decrease in the growth response of intact squash hypocotyls after the third day. The change in the concentration of IAA in the SY solution was, however, smaller than that in the AP solution. These results suggest that the hypocotyl growth is related to the concentration of IAA in the apoplast rather than in the symplast.

The concentration of IAA in the AP solution was immediately increased upon treatment of segments with exogenous IAA, and the concentration reached 75% of the exogenous concentration in 4 h. IAA was not detectable in the apoplast when segments had been incubated in water for 2 h. Therefore, IAA might have leaked from the apoplast to the ambient water or might have been absorbed by cells, probably moving freely in the apoplast. Cosgrove and Cleland (1983) reported that the AP solution was exchanged with the ambient solution for about 45 min in experiments to monitor diffusion of mannitol from segments of several plants. Yamamoto and Sakurai (1992) also reported that AP solution was exchanged with the exogenous solution for about 1 h in experiments designed to monitor diffusion of non-radioactive and radioactive mannitol from pea segments. These reports support our results for the rate of entry of IAA into the apoplast in our experiments and also demonstrate the validity of our centrifugation method for collection of AP solution.

We can not exclude the possibility that IAA diffuses from the symplast to the apoplast in squash hypocotyls, but the fact that the concentration of IAA in the symplast of intact segments was similar to that in the symplast of segments that had been incubated in water for 2 h might exclude the possibility of the rapid outward transport of IAA across the plasma membrane, driven by an energy-consuming mechanism that involves an ATPase.

In this study, we revealed the unequal distribution of IAA in AP and SY solutions. The SY solution is thought to include vacuolar contents. IAA is unlikely to enter vacuoles, since IAA dissociates in the cytoplasm. Vacuoles in mature cells account for about 90% of the cell volume, but the vacuolar volume in younger, growing cells is not known. If increases in cell volume depend on increases in vacuolar volume, while the cytoplasmic volume is constant, in upper and lower parts of hypocotyls, we can calculate the vacuolar volume at a given stage by measuring cell volume. The epidermal cells in the upper, growing part were about 55 μm long and those in the lower, mature part

were about 163 μm long. The vacuolar volume of cells in the upper part of the hypocotyl was estimated to be about 70% of the cell volume. If IAA is not present in vacuoles but is only found in the cytoplasm, the concentration of IAA in the SY solution in the upper part of 2-d-old hypocotyls can be calculated to be 16.3×10^{-8} M (4.90×10^{-8} M $\times 100\%/30\%$) from the data in Table 2, which is still lower than the concentration in the apoplast (22.6×10^{-8} M). If the assumption that IAA is present only in the cytoplasm is correct, the concentration of IAA in the symplast in the lower part of 2-d-old hypocotyls would be 27.1×10^{-8} M which is higher than that in the symplast in the upper part (17.9×10^{-8} M). In other words, mature cells would contain a higher concentration of IAA in their cytoplasm than younger, immature cells. This discrepancy should be clarified by separate measurements of levels of IAA in the cytoplasm and the vacuole.

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