

Immunogold-EM analysis reveal brefeldin a-sensitive clusters of auxin in Arabidopsis root apex cells

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ABSTRACT

Immunogold electron microscopy (EM) study of Arabidopsis root apices analyzed using specific IAA antibody and high-pressure freeze fixation technique allowed, for the first time, visualization of subcellular localization of IAA in cells assembled intactly within plant tissues. Our quantitative analysis reveals that there is considerable portion of IAA gold particles that clusters within vesicles and membraneous compartments in all root apex cells. There are clear tissue-specific and developmental differences of clustered IAA in root apices. These findings have significant consequences for our understanding of this small molecule which is controlling plant growth, development and behavior.

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Introduction


Indole-3-acetic acid (IAA or auxin) is considered for a plant hormone, but it has also morphogen and signaling transmitter features. IAA is involved in almost all plant processes during the entire life span of higher plants. After it was predicted by Charles and Francis Darwin in 1880,¹ this small signaling molecule was discovered and initially characterized by Went, Kögl, Thimann and others in the first half of the last century.² Boysen-Jensen confirmed that a mobile signal molecule is involved in phototropism in 1913³ and Went coined term auxin (from Greek *auxein*) in 1926,⁴ which was then chemically identified as IAA in 1934.⁵ Finally, IAA was isolated from immature maize seeds in 1946.⁶

Charles and Francis Darwin, in their experiments with decapped maize roots, followed original experiments accomplished by Theophil Ciesielski in 1872 who discovered that decapped maize roots continue to grow but fail to respond to gravistimulation.⁷ Julius Sachs heavily criticized these experiments but they were confirmed later by Francis Darwin as well as many other experimental researchers.⁸⁻¹⁵ Currently, IAA and its transcellular transport are well known to be essential for all kinds of plant tropisms, both in roots and shoots. Especially root apices are unique as almost all PIN proteins are known to be

expressed in root apex of Arabidopsis and drive the very complex loops of transcellular auxin fluxes.

IAA is very small molecule (molar mass 175.19 g·mol⁻¹), which can rapidly diffuse through the cytoplasm as well as through plant cell walls, that affects and controls almost every aspect of plant biology.^{16,17} Surprisingly, although auxin easily diffuse into nuclei through their nuclear pores to control gene expression, it is not freely transported through larger plasmodesmata.^{18,19} Either plasmodesmal gating excludes auxin from passing through plasmodesmata or there is some active mechanism behind, which prevents IAA to enter plasmodesmata. Obviously, this is an IAA specific process as synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D)²⁰ is passing through via plasmodesmata.²¹ One possible scenario would be that putative plasma membrane derived recycling vesicles, internalizing IAA via activity of the PIN efflux transporters, *patrol* plasmodesmata orifices and actively prevent IAA molecules to enter the plasmodesmata channels. 2,4-D molecules, which are not transported via auxin efflux carriers,^{20,22} are allowed to pass along plasmodesmata. In support of this concept, IAA is known to accumulate within plasma membrane-derived vesicles via an active electrogenic transport of IAA.²³ Moreover, immunolocalization of IAA using

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specific antibodies revealed IAA-enriched vesicles in maize root apex cells, co-localizing with recycling auxin efflux transporter PIN1 and with endosomal recycling pectins.^{24,25} However, at low resolution of the light immunofluorescence microscopy, it is not possible to reach any conclusive evidence on auxin molecules accumulating within the lumen of recycling vesicles. To localize auxin molecules in cells of plant tissues, the only possible method is immunogold electron microscopy (EM) using specific antibodies. EM immunolocalization of low molecular weight compounds (slightly below of that of IAA's 175,18 Da) have already been reported using anti-GABA and L-glutamate antibodies applied on fixed rat brain sections.^{26,27} Both GABA and L-glutamate are localized in synaptic vesicles with higher density (clusters) as in the adjacent cytoplasm.^{26,27}

Results

Here, we have used immunogold electron microscopy (EM) analysis of Arabidopsis root apices fixed using high-pressure freeze fixation technique which preserves membranes, organelles and ultrastructure of the fixed cells. Our analysis revealed that all cells of Arabidopsis root apices show auxin-labeled gold particles clustered within vesicular structures (Fig. 1, 2 and 3). Most of them were tightly clustered, from 3 up to about 80 gold-particles within one single compartment. Importantly, there are both tissue-specific and developmental differences, with large auxin clusters scored especially in root cap and root epidermis cells (Fig. 3A). There were almost no auxin clusters scored within quiescent center cells, and only small clusters in the adjacent initial cells. Size of IAA clusters is increasing along the root apex, reaching a peak in the transition zone, and then decreasing in cells of the early elongation zone (Figs. 1, 2). The number of clusters is high in the

root cap and the transition zone cells, while no or very few IAA clusters are in the quiescent center and adjacent initial cells (Figs. 1, 2). When the first antibody was omitted then no gold particles were present (Fig. 3B).

Interestingly, this pattern of clustered IAA, shown here for both IAA antibodies, closely mimicks rates of endocytic vesicle recycling in the root apex zone, being the highest in the transition zone cells, both in maize and Arabidopsis.^{24,28} Clusters of IAA were scored from 3 up to 80 gold particles with both IAA antibodies (Figs 1, 2). Interestingly, exposure of Arabidopsis roots to inhibitor of exocytosis and endocytic vesicle recycling Brefeldin A (BFA) resulted in loss of IAA clusters in all root cap and elongation region cells, whereas the size of clusters decreased in meristem and transition zone cells (Figs. 1, 2)

Discussion

Current models of transcellular auxin transport are based on membrane transporters of PIN and ABC families which are considered to be active as transporters only when inserted in the plasma membrane. However, these auxin transporters are actively recycling between the plasma membrane and endosomes via recycling vesicles.²⁹⁻³¹ Importantly, there is no evidence available, whatsoever, that these transporters stop to transport IAA when localized within the vesicle membrane. In fact, some authors insert PIN arrows (indicating their auxin transport activities) also into the vesicle and endosomal membranes in their schemes.³²⁻³⁵ Our present data strongly support this scenario and PINs represent the best candidate for vesicular auxin transporters.

There are several features of the polar auxin transport (PAT) implicating exocytic secretion of auxin.^{28,36,37} First of all, there is tight correlation between *exo/*

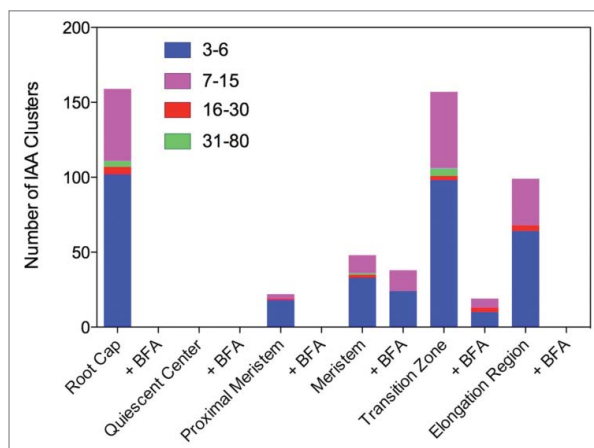


Figure 1. Quantification of gold particle distributions in root apex zones using the polyclonal IAA antibody.

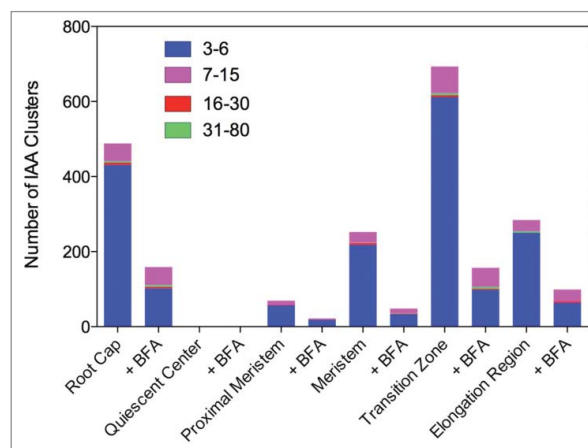


Figure 2. Quantification of gold particle distributions in root apex zones using the monoclonal IAA antibody.

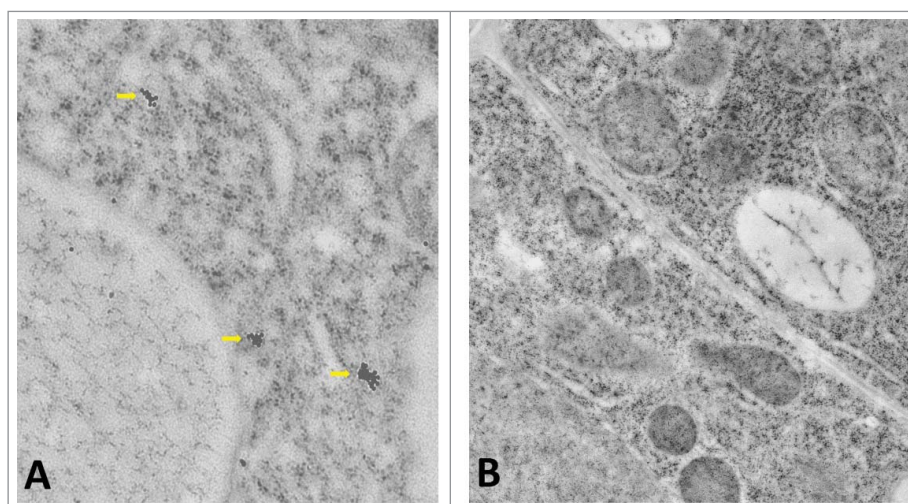


Figure 3. (A) Three IAA clusters in a transition zone epidermis cell labeled with the polyclonal IAA antibody. (B) Negative control of similar cell using only the secondary antibody results in no gold particles.

endocytic vesicle recycling and rate of the PAT. Second, inhibitors of PAT inhibit endocytic vesicle recycling whereas exocytosis inhibitor brefeldin A (BFA) inhibits endocytic vesicle recycling and PAT.³⁸⁻⁴¹ Third, amounts of PINs inserted within plasma membrane do not correlate with rates of PAT.³⁶ Importantly, BFA mimicking PAT transport inhibitors also similarly affects gravistimulation-induced calcium spikes in Arabidopsis seedlings.⁴²

Recently, vesicular secretion of IAA via single exocytic vesicle fusion was even experimentally confirmed with *in situ* core-shell titanium-carbide carbon (TiC&C) quasi-aligned nanofiber arrays (QANFAs). Quantal secretion of IAA using this amperometric monitoring system of IAA release allows real time quantification of IAA release.⁴³ Importantly, the quantal size of IAA secretion events was increased by pre-loading of plant cells with IAA molecules.⁴³ The same system was used also for quantification of GABA and L-glutamate release at brain synapses.⁴⁴⁻⁴⁶ This depolarization-induced IAA secretion release from plant protoplasts (Fig. 4)⁴³ closely resembles neurotransmitter vesicular release of GABA and L-glutamate at the neuronal synapses. As IAA is well-known to induce electrical responses, and even plant action potentials, all this strongly supports the neurotransmitter-like concept of auxin actions in plants.^{24,28,36,37}

Interestingly, there is a clear negative correlation between vesicular auxin and auxin-mediated gene expression⁴⁷ as well as auxin response maximum mediated via synthetic auxin-response reporter DR5,⁴⁸ and PLETHORA transcription factor gradient in Arabidopsis root apex.^{49,50} Our data suggest that auxin-accumulating vesicles control how much IAA molecules are free, available to enter nuclei for the auxin induced gene expression.

IAA enriched vesicles and/or endosomes may correspond to the mysterious compartment X proposed by Markus Grebe⁵¹ to explain role of intracellular proton pump AVP1 in control of PAT and cell wall acidification.^{52,53} AVP1 belongs to pyrophosphatases which localize to trans-Golgi networks and multivesicular bodies,^{54,55} both being part of the plant endosomal system. Importantly, AVPs are localized within the BFA-induced compartments⁵⁵ in which also IAA and PINs are enriched.^{24,25} More recently, the possible input of AVP1 into PAT was

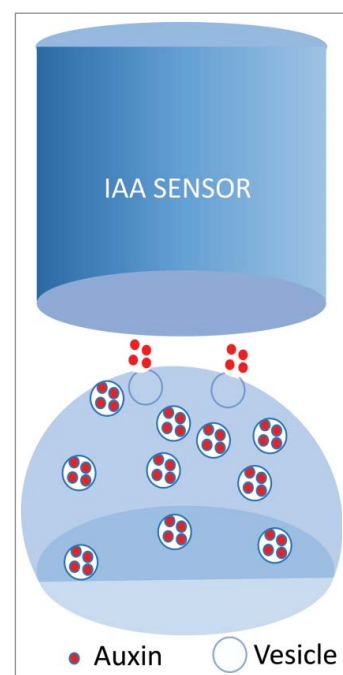


Figure 4. Plasma membrane depolarization-induces quantal IAA exocytosis from plant protoplasts probed with auxin-specific micro-electrochemical sensor.⁴³

toned down due to a second T-DNA insertion close to the ARF-GEF GNOM gene in the *avp1-1* mutant line.^{56,57} It is relevant, however, that GNOM is plant-specific ARF-GEF which is mediating BFA-mediated endosomal recycling-based PAT.⁵⁸⁻⁶⁰ Both GNOM and AVP1 localize to BFA-sensitive endosomal compartments accumulating within the BFA-induced compartments.

Interestingly in this respect, our data show that the BFA exposure induces loss of almost all vesicular IAA in root cap cells as well as elongating root cells, which are rather inactive in the endosomal vesicle recycling and PAT; but only decrease in cells of meristem and transition zone which are active in the endosomal vesicle recycling and PAT.^{24,28,36,37,61-63} Several aspects of the aluminum (Al) toxicity of the root apex transition zone support the emerging concept that the recycling auxin-accumulating vesicles control how much auxin is available for nuclear transport to control gene expression. Similarly as BFA, Al toxicity inhibits endocytic vesicle recycling and PAT in the transition zone of both maize and Arabidopsis root apices.⁶⁴⁻⁶⁷ Moreover, Al induces IAA accumulation within nuclei of the Arabidopsis root apex transition zone, as visualized with the DNA transcription-based IAA reporter DR5.^{68,69}

Conclusions

In conclusion, the EM immunogold visualization of IAA in Arabidopsis root apex cells is incompatible with the currently popular models of PAT considering auxin to be diffusely distributed within the cytoplasm and ignoring endosomes / vesicles in the PAT. Together with the recent discovery of vesicular secretion of IAA from protoplasts, our data implicate vesicular secretion of IAA in the PAT. This feature has far-reaching consequences not only for auxin biology but also, as IAA affects all aspects of plant biology,^{16,17} for our understanding of plants in their sensory and behavioral complexity. PAT is essential for all kinds of plant movements and tropisms,^{15,28-37} as well as for integrating plant bodies via long-range IAA fluxes via vascular systems⁷⁰ and short-range IAA fluxes.⁷¹ Ability to actively enrich IAA within vesicles and larger compartments is important for plant growth, polarity and development as it allows plant cells to control precisely when and how much IAA molecules are reaching their signaling targets and receptors, controlling plant signaling at the plasma membrane and gene expression levels within the nucleus. Quantal release of IAA from cells is also critical for IAA acting as transmitter molecule controlling cell-cell communication via neuronal-like processes.^{72,73} Last but not least, the secretion of auxin via recycling vesicles can solve the mystery of the

elusive flux sensor which is required for the PAT models based on IAA canalization.^{35,74} All published models of PAT ignore IAA enriched vesicles and endosomes. This is a serious drawback of these models, limiting their reliability and predictability. If the neurotransmitter-like recycling of the IAA-enriched vesicles^{24,72,73} will be confirmed in future, this will represent significant breakthrough not only in auxin biology and physiology, but also in plant sensory and behavioral biology. Relevantly in this respect, recent support for roles of vesicles in PAT emerges from studies of large algal cells of Chara,⁷⁵ plant-specific myosins,^{76,77,78,79} and ER-dependent endosome streaming via RHD3 protein⁸⁰ expressed highly in the root apex transition zone.⁸¹

Materials and methods

Root apices of 7 d old control and BFA-treated (50 μ M BFA for 2 hours) Arabidopsis seedlings were used. Immunogold labeling was performed as previously reported.⁸²⁻⁸⁴

Three root apices were placed in gold platelet carriers pre-filled with 1-hexadecene and immediately frozen in a high-pressure freezing apparatus (HPM100; Bal-Tec). Subsequently, samples were chemically fixed with 0,1% uranyl acetate and 0, 25% glutaraldehyde in dry acetone. Then, samples were cryosubstituted at -80°C and embedded in Lowicryl HM20 (Polysciences, Warrington PA). Ultrathin sections were blocked and incubated in a moist chamber with primary antibodies (1:200) diluted overnight at 4°C . Ultrathin sections were blocked against binding to unspecific proteins, post-fixed with 2% glutaraldehyde, and post-stained with 2% uranyl acetate. Ultrathin sections were incubated primary IAA antibodies diluted 1:200 (affinity purified anti-N1-IAA; polyclonal)^{24,85} or 1:200 (anti-IAA monoclonal 1E11-C11, Sigma-Aldrich) overnight at 4°C . This step was followed by rinsing and incubation with secondary antibodies conjugated to 10 nm gold particles. Secondary antibodies were diluted 1:50 with PBS and incubated for 2 h at room temperature. Controls were performed using only the secondary antibodies conjugated to 10 nm gold particles. Labeled sections were examined with an Leo 912ab transmission electron microscope (Zeiss) operated at 80 keV. For BFA treatments, the roots were incubated with 35.6 μ M BFA at room temperature for 60 min. Clusters of auxin-labeled gold particles were quantified across the examined root apex tissues/zones and were not calculated at the cellular level.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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