Substantial Evidence for Auxin Secretory Vesicles

Dear Editor,

In their letter, Robinson et al., 2018 question our recent report on auxin clustered within cells of Arabidopsis root apices. They claim that small molecules such as IAA cannot be visualized using the immunogold EM techniques. However, in 2006, we succeeded in immunolocalizing IAA via light microscopy using Steedman's wax sections in maize root apices (Schlicht et al., 2006). Now, using high-pressure fixation probes, we have achieved very interesting EM immunogold immunolocalization of IAA using Arabidopsis root apices (Mettbach et al., 2017). Although the EM probes do not allow a clear visualization of limiting membranes—which is often the case of EM immunogold analysis in turgescent plant cells—the patterns of the IAA clusters scored (ranging from three to 80 clusters of 10-nm gold particles) suggest that these include not only the endosomes but also the recycling vesicles. It is not so surprising that IAA localization in maize coleoptiles cells is not vesicular as elongating cells of the Arabidopsis root apex also do not show prominently clustered auxin (Mettbach et al., 2017). Both IAA antibodies used in EM immunogold immunolocalization were extensively checked for specificity (for polyclonal N-IAA, see Schlicht et al., 2006; for monoclonal 1E11-C11, see e.g. Moctezuma, 1999 and many others). Negative control, provided by omitting of the first antibodies and using only the secondary antibodies, resulted in no gold particles (Mettbach et al., 2017).

Robinson et al. are right that we cannot be sure about the membranous nature of these gold particle clusters. However, there are several features strongly suggesting that they are specific and, in fact, based on vesicles and compartments. First of all, there are tissue-specific patterns when, for example, cells of the quiescent center never showed any gold clusters. Second, there also are clear developmental trends when cells from the elongation region only rarely showed clusters of gold particles. On the other hand, the highest numbers of clustered gold particles were scored from cells in the root apex transition zone. Finally, IAA-based gold particle clusters are sensitive to inhibitor of exocytosis BFA and vanished completely from the root cap cells. Interestingly, IAA gold cluster numbers decreased significantly in root apex cells of the BFA-exposed roots. Maximal peaks of in vivo IAA fluxes, sensitive to BFA and polar auxin transport inhibitors, were reported in the root apex transition zone with the IAA-specific microelectrodes (Mancuso et al., 2005, 2007; McLamore et al., 2010). This congruence between auxin clustering (implying vesicular auxin) and in vivo auxin fluxes in the root apex transition zone is very intriguing.

Importantly, Liu et al. (2014) reported vesicle-based quantal secretion of IAA from plant protoplasts directly via electrochemical vesicle cytometry (Liu et al., 2014). This new in vivo amperometry technique provides an excellent ultrafast tool to probe quantitatively vesicle-based exocytosis events (Liu et al., 2014). We provided genetic evidence for the synaptic-like auxin fluxes peaking in cells of the Arabidopsis root apex transition zone via phospholipase D Zeta2 mutants (Mancuso et al., 2007). Free auxin minimum scored with in vivo fluorescent reporters (Di Mambro et al., 2017) coincides with the vesicular auxin maximum, which is closely related to endogenous auxin fluxes (Mancuso et al., 2005, 2007; McLamore et al., 2010). It is possible that the free auxin minimum in cells of the root apex transition zone (Di Mambro et al., 2017) is generated by vesicular auxin transporters that deplete the cytoplasmic free auxin by transporting auxin into vesicular lumens. Finally, active carrier-mediated uptake of auxin into vesicles was well characterized biochemically in the 1970s by the groups of Rainer Hertel and Winslow Briggs (Hertel et al., 1983; Lomax et al., 1985).

With respect to the claims that it is not possible, in principle, to localize such a small molecule using the EM immunogold immunolocalization technique, we have cited recent reports on EM immunogold analysis of neurotransmitters in similar size range as IAA (GABA, Glu, and other amino acids; Bergersen et al., 2012). Importantly, in our hands, IAA localization in root apex cells does not require the protein cross-linker EDAC fixation, which has potential side effects. In fact, we deliberately have not used EDAC fixation in our immunofluorescence localization of IAA (Schlicht et al., 2006) as our unpublished data revealed loss of F-actin from samples fixed with EDAC (F. Baluška, unpublished data). We consider the dense meshworks of pectin matrix, when RGII pectins are cross-linked with boron and RGI pectins with calcium within endosomes and recycling vesicles (Šamaj et al., 2004), to be relevant for the retention of IAA molecules within these compartments. Importantly, using the exact same high-pressure probes we have used for the EM immunogold localization of IAA (Mettbach et al., 2017), we observed MPK6 was associated with vesicles and TGN/endosomal compartments (Müller et al., 2010), whereas Flotillin 1 localized preferentially to the plasma membrane (Li et al., 2012). Importantly, also using the identical probes, we did not score prominently clustered gold particles using the MPK6 and Flotillin 1 antibodies in our previous EM immunogold localizations (Müller et al., 2010; Li et al., 2012).

We agree that we require unequivocal evidences to be sure about the roles of endocytic vesicle recycling in the polar auxin transport. In this respect, we would like to comment briefly about the significant report that all auxin transport inhibitors block the endocytic vesicle

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recycling, while BFA-compromised vesicle recycling inhibits the polar auxin transport in root apex cells of Arabidopsis (Geldner et al., 2001). Moreover, endosomal GNOM ARF-GEF drives polar auxin transport via vesicle recycling (Geldner et al., 2003). Claims about the PINs transporting IAA only when inserted within the plasma membrane, but not when inserted within the membranes of endosome and recycling vesicles (see Robinson et al., 2018), have never been substantiated by unequivocal and water-proof evidence. All these critical issues must await further cytological and biochemical studies.

František Baluška, Miroslav Strnad, and Stefano Mancuso

František Baluška
IZMB, University of Bonn, Bonn, Germany

Miroslav Strnad
Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany AS CR and Palacký University, Olomouc, Czech Republic

Stefano Mancuso
Department of Plant, Soil, and Environmental Science and LINV, University of Florence, Sesto Fiorentino, Italy

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