Internalized and Newly Synthesized *Arabidopsis* PIN-FORMED2 Pass through Brefeldin A Compartments: A New Insight into Intracellular Dynamics of the Protein by Using the Photoconvertible Fluorescence Protein Dendra2 as a Tag

Dear Editor,

Plasma membrane (PM)–localized PIN-FORMED (PIN) auxin efflux carriers were shown to cycle rapidly and continuously between PM and the endomembrane system and this cycling is affected by many exogenous factors and endogenous programs (Grunewald and Friml, 2010). The fungal lactone metabolite Brefeldin A (BFA) is believed to interrupt the continuous cycling of PINs and other PM proteins by inhibiting their re-secretion (Kleine-Vehn and Friml, 2008). Proteins are congregated in a large vesicular structure called BFA compartments (BFACs; Robinson et al., 2008). In many studies, the failure to detect PIN-labeled BFACs was considered a sufficient sign of PINs endocytosis inhibition (Paciorek et al., 2005; Dhonukshe et al., 2007; Robert et al., 2010). However, the activity of BFA on plant cells seems to be more complex. The results obtained using different organelle markers imply that distinct populations of aggregates induced by BFA application might exist and that clustering of different subcellular structures depends on the concentration and duration of BFA exposure (Robinson et al., 2008; Lam et al., 2009a, 2009b).

PINs in BFACs can be visualized either by using fluorescent tags or by immunocytochemistry but quantitative data obtained by these methods show only the net abundance of proteins and do not allow direct and simultaneous monitoring of both internalized and newly synthesized protein pools. Recently, we used a new approach employing the green-to-red photoconvertible fluorescent protein Dendra2 to study the turnover of PIN2 in PM (Jásik et al., 2013). In experiments with BFA applying the previously established method for plant growth, microscopic imaging, and photoconversion of Dendra2 (Jásik et al., 2013 and methods in Supplementary Data online), we noticed that BFACs contained both the red PIN2–Dendra2 variety originating from PM and the green form representing the newly synthesized pool (Supplemental Figure 1). This unexpected finding prompted us to examine in more detail the influence of BFA on dynamics of PIN2 populations.

Because the spectral properties of Dendra2 are pH-sensitive (Jásik et al., 2013), we asked at first whether the fluorescence emission is affected by the subcellular localization of PIN2–Dendra2. Spectra emitted by PM and BFACs showed identical patterns (Supplemental Figure 2), so we concluded that Dendra2 is a reliable tool for quantitative analysis of fluorescence intensities in those two subcellular structures. We then proved that PIN2–Dendra2 behaves similarly to the commonly used PIN2–GFP fusion (Xu and Scheres, 2005) employed in many previous studies. We analyzed parameters of BFACs frequently evaluated in studies on PINs internalization and demonstrated standard trafficking of PIN2–Dendra2 through the BFA-sensitive pathway (Supplemental Figure 3).

Previous studies suggested that effects of BFA might be concentration-dependent (Lam et al., 2009a). Therefore, we investigated how dynamics of PIN2–Dendra2 in BFACs and PM were affected by different doses of BFA. We found a slight attenuation of red signal disappearance from PM and a considerable suppression of green signal recovery in PM in a clearly dose-dependent manner (Figure 1A, upper panel). This finding is surprising because, if BFA restrains re-secretion of PINs back to PM (Kleine-Vehn and Friml, 2008; Grunewald and Friml, 2010), one should expect rapid vanishing of the red signal from PM. The BFA concentration has also a strong impact on the green-to-red ratio in BFACs; the red form predominated at 200 μM while the green one prevailed at lower concentrations (20 and 50 μM) after 3 h (Figure 1A, lower panel). Furthermore, a time course study (Supplemental Figure 1) and detailed imaging after 4 h BFA treatment (Figure 1B) demonstrated that BFACs and PM showed a different PIN2 turnover rate. This result was confirmed with seedlings grown for 2 h under BFA treatment and having fully developed BFACs. Shortly after conversion, the patterns of spectra emitted by PM and BFACs were identical but later the red signal disappeared more quickly from BFACs than from PM (Figure 1C, left panel, quantitative data are in right panel). We conclude that BFACs are

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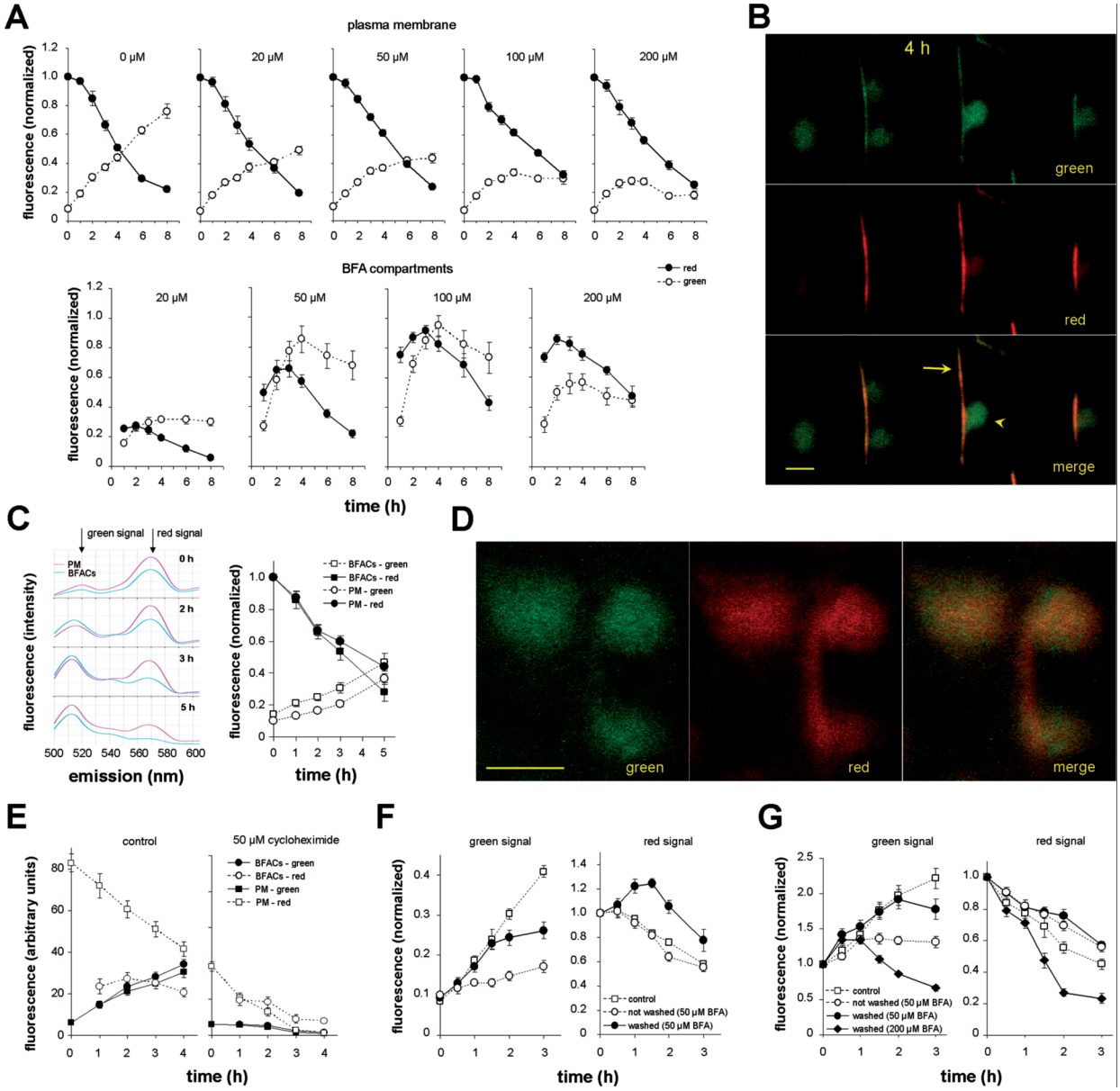

Figure 1 Dynamics of PIN2 Populations in BFACs and PM.

(A) Effect of different BFA concentrations on the PIN2 turnover in PM and BFACs.

(B) Predominance of the green signal over the red signal in BFACs (arrowhead), but not in PM (arrow) after 4 h BFA treatment. Scale bars = 5 μm.

(C) Time course spectral analysis by the meta detector, the lambda acquisition mode and 488-nm laser line (left panel), and quantitative data on PIN2 turnover in PM and BFACs obtained by analysis of standard images (right panel). Roots were treated for 2 h with BFA.

(D) BFACs developed within 2 h after conversion. Scale bars = 5 μm.

(E) Effect of CHX on PIN2 abundance in BFACs and PM. Roots were photoconverted and then seedlings were treated with CHX for 90 min then transferred on the medium with BFA and CHX for a time course analysis. Control seedlings were not treated with CHX.

(F) Dynamics of red and green signals in PM after BFA washing-out. Before time course analysis, seedlings were treated with BFA for 2.5 h, rinsed for 10 s with liquid culture medium, placed on BFA-free medium (marked as 'washed'), imaged, photoconverted, and reimaged. In parallel analysis, the seedlings were kept on medium either with (marked as 'not washed') or without BFA (marked as 'control') during the whole experiment.

(G) Impact of green and red pool of PIN2–Dendra2 released from BFACs after washing on PIN2 pools abundance in PM. Before the time course analysis, roots were photoconverted then seedlings were kept on medium with BFA for 2.5 h, washed, and placed on medium without BFA. Corresponding controls were grown continuously on medium either with BFA (marked as 'not washed') or without BFA (marked as 'control').

dynamic structures and both PIN2–Dendra2 varieties are passing through them before reaching their final destinations. In previous studies, BFACs were regarded as somehow static structures where internalized proteins are retained on their traffic route back to PM (Kleine-Vehn and Friml, 2008).

In *Arabidopsis* root tips, BFACs are formed by large vesicle aggregates surrounded by Golgi stacks (Robinson et al., 2008). As newly synthesized and internalized PIN2 populations are processed in various ways, we examined whether this is reflected by the distribution of both PIN2–Dendra2 varieties within BFACs. Our analysis showed no clear segmentation of the green and red signal in BFACs, such as accumulation of a specific variety in the core or at the periphery of BFACs (Figure 1D).

To analyze in a short time a sufficient number of cells in individual roots, we routinely photoconverted whole root tips. In this way, the newly synthesized pool which is still not delivered to PM is also converted to the red form and consequently the red variety in BFACs may represent the newly synthesized pool and not the internalized population. In order to dispel any doubt, we performed a series of experiments with cycloheximidine (CHX) and actinomycin. As expected, after photoconversion and 90-min CHX pretreatment, no green signal was detected either in PM or BFACs; however, the red signal was still present in both structures (Figure 1E). This finding was confirmed by a modified test (Supplemental Figure 4). Altogether, these experiments again confirm that both pools of PIN2–Dendra2 traffic through a BFA-sensitive pathway.

Major evidence supporting a permanent cycling of PINs between PM and the endomembrane system comes from washing-out experiments (Geldner et al., 2001). When we rinsed seedlings that had been cultivated on BFA medium for 2.5 h and then photoconverted them, we could confirm the disappearance of BFACs from cells (Supplemental Figure 5) and the transient increase of red signal intensity in PM (Figure 1F). Quantitative analysis in the parallel experiment with unconverted samples showed an ~30% increase in signal intensity in PM (Supplemental Figure 6). These results suggest that at least some portion of PIN2–Dendra2 should be relocated from BFACs to PM. According to the concept of PINs recycling, it should be the internalized PIN2–Dendra2 pool of PM origin (Kleine-Vehn and Friml, 2008). However, the presence of a newly synthesized PIN2–Dendra2 population in BFACs challenges this statement. In order to adequately address this question, we performed a modified washing-out experiment in that seedlings were initially photoconverted and then treated with BFA. After 2.5-h BFA treatment, green and red signal intensities in BFACs are roughly in equilibrium (Figure 1A and 1D). After washing, we observed an accelerated increase of green signal intensity in PM but we failed to detect an increase in red signal intensity (Figure 1G). We confirmed this finding with the high BFA dose of 200 μM (Figure 1G) upon which BFACs contain relatively high levels of the red PIN2–Dendra2 variety (Figure 1A). Finally, in another approach, we photoconverted roots and allowed them to develop BFACs in the presence of CHX. As expected, BFACs contained only red PIN2 variety of PM origin. After rinsing, BFACs disappeared from cells but we were not able to detect simultaneous increasing red signal intensity in PM (Supplemental Figure 7). We concluded that only newly synthesized PIN2 is trafficking from BFACs to PM. Red form of PIN2–Dendra2 temporally gathered in BFACs is most likely targeted to the vacuole for degradation. Indeed, the shuttling of PIN2 and other proteins from BFACs to vacuoles is well documented (see Grunewald and Friml, 2010, for review). These experiments again do not confirm PIN2 recycling. In the study that is considered to be the direct evidence of PIN2 continuous cycling, Dhonukshe et al. (2007) used the green-to-red photoconvertible fluorescence protein EosFP and, after selective photoconversion of PM, they observed the appearance of a red signal in BFACs. Alternatively, after conversion of BFACs and BFA removing, they recorded the red signal in PM. However, in these separated experiments, the authors have not considered a double origin of PIN2 located in BFACs, they have not tracked simultaneously both the secretory and internalized PIN2 populations, and thus they did not really demonstrate continuous PIN2 recycling.

The results described above suggest that the appearance/absence of PIN2 in BFACs is not an appropriate method to monitor PIN2 endocytosis. To support this statement, we performed a series of experiments with Tyrphostin A23 (A23). Dhonukshe et al. (2007) demonstrated the inhibitory effect of this compound on the endocytosis of several plant transmembrane proteins indirectly by monitoring their appearance in BFACs. We observed the subtle deceleration in the decay of red signal intensity in PM after A23 treatment (Supplemental Figure 8A) but this effect was only temporary despite the application of a relatively high dose of A23. The simultaneous analysis of the green signal showed a strong inhibitory effect of A23 on green signal recovery in PM (Supplemental Figure 8A). When samples were pretreated by A23 and then treated by BFA, BFACs were clearly observable in both channels. They emitted red signals with slightly reduced intensity in comparison with control samples but their green signal intensity was diminished considerably (Supplemental Figure 8B). Considering the previous study by Dhonukshe et al. (2007), one should expect to find no red fluoresce in BFACs. Our result implies that the reduction of signal intensity in BFACs observed in the experiments with the endocytosis inhibitor A23 using GFP as a tag or by immunocytochemistry could be caused rather by inhibition of delivery of newly synthesized protein than by inhibition of protein internalization. It cannot be excluded that also other factors previously studied by the BFA approach and assumed to affect endocytosis of PIN2 may operate actually through its synthesis and/or secretion pathways.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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Ján Jásik[a–c,1] and *Elmon Schmelzer*[a]

a Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829, Köln, Germany
b Department of Botany, Révová 39, Faculty of Natural Sciences, Comenius University, 811 02 Bratislava 1, Slovakia
c Department of Molecular Biology, Mlynská dolina, pavilion B-2, Faculty of Natural Sciences, Comenius University, 842 15 Bratislava 4, Slovakia
[1] To whom correspondence should be addressed at address c. E-mail jan.jasik@rec.uniba.sk, tel. +421 (02) 602 96 454, fax. +421(02) 654 29 064.

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