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Influx and Efflux of Strigolactones are Actively Regulated and involve the Cell Trafficking System

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Dear Editor,

Strigolactones (SLs) are plant hormones that regulate different aspects of plant development. In roots, SLs are involved in the regulation of lateral-root formation and they induce root-hair elongation. They are also exuded from plant roots and act as stimulators of parasitic and symbiotic (e.g., arbuscular mycorrhizae) interactions. SLs are perceived in plants by a specific receptor system that consists of several interacting proteins (reviewed by Al-Babili and Bournmeester, 2015).

A putative transporter of SLs was previously identified in petunia (Petunia hybrida): the ATP-binding cassette (ABC) protein designated PDR1. It was shown to have a key role in petunia in regulating the development of arbuscular mycorrhizae and auxiliary branches, by functioning as a cellular SL exporter (Kretzschmar et al., 2012). pdr1 mutants were aberrant in symbiotic interactions and shoot phenotype, suggesting impaired SL allocation. In Arabidopsis thaliana overexpressing Petunia axillaris PDR1, tolerance to high concentrations of a synthetic SL was enhanced, suggesting increased export of SLs from the roots (Kretzschmar et al., 2012).

However, only little is known about the movement of SLs, their precursors or their derivatives in general, and in Arabidopsis in particular.

Herein we present new evidence for SL distribution in the plant using fluorescent SL derivatives. Among the different fluorescently tagged SL-like compounds synthesized and tested in our laboratories, fluorescent BODIPY (BP)-tagged SL analogs have the desired bioactivity and spectroscopic properties (Prandi et al., 2014). Also, the SL analog EGO10 sharing the core structure with EGO10-BP, has been shown to act on the root to increase root-hair length (Cohen et al., 2013). Therefore, we synthesized EGO10-BP which is the SL analog EGO10 functionalized with green BP as the fluorophore (Supplemental Figure 1) by means of a 3 C linker. Four different BP derivatives differing in their structure were used: EGO10A-BP (pure enantiomer A), EGO10B-BP (pure enantiomer B), EGO10-md-BP (SL analog EGO10-BP lacking the D-ring and the enol ether bridge) and naked-BP (fluorophore only). The EGO10-BP serie presents a simplified stereochemistry with respect to natural SLs as only a stereocenter is present at the C-2' position. Using CD spectra and chiral HPLC behaviour (not shown) EGO10A-BP was established to be the
enantiomer with natural SL structures (R configuration), conforming with canonical
SLs structure.

Agar cubes containing fluorescent SLs (EGO10A-BP, EGO10B-BP, or EGO10-
mD-BP or naked-BP (at 10 µM concentration) were placed on Arabidopsis seedling
roots (Supplemental Material and Methods and Supplemental Figure 2). Fluorescent
signal was detected in the treated roots after 24 h and quantified (using IMAGEJ) in 9
segments of 100 µm each (Supplemental Figure 2), shoot-ward or root-ward from the
agar cube placement.

The naked-BP treatment resulted in a very high signal in the root tissue
(Supplemental Figure 3). The EGO10A-BP signal was significantly higher than that
of EGO10B-BP or EGO10-mD-BP, both shoot-ward (Figure 1A) and root-ward
(Supplemental Figure 3). Accordingly, EGO10A-BP was the most active analog for
root hair elongation in root segments above the agar cube compared to the other
compounds tested (Figure 1B), similarly to the activity of EGO10A (Supplemental
Figure 4). Also, placement of agar cube without any SL analog does not significantly
change root hair length in comparison to non-treated control (Supplemental Figure 4).
Although shoot-ward signal was slightly higher than that of the root-ward, no
significant differences between the two were detected (Supplemental Figure 3).

At the cellular level, EGO10A-BP signal was detected in the root epidermis and
to a much lesser extent in root cortex and vascular tissues (Figure 1C). The naked-BP
molecule signal was high in all root tissues: it was apparent mostly in the root cortex
and vascular tissues, and to a relatively lesser extent in the root epidermis (Figure 1C).
At the subcellular level, EGO10A-BP signal was detected in the cytoplasm, in
vesicle-like bodies, in endosomal-like structures and in correspondence of nuclei,
present to a smaller extent in the nucleus and labelling the nuclear envelope (Figure
1C; additional examples for images in Supplementary Fig 5). Since EGO10A-BP was
biologically active (Figure 1B), it might be that the its relatively small extent in the
nucleus was sufficient to acknowledge activity. The naked-BP molecule signal was
distributed evenly across the cell cytoplasm (Figure 1C) and did not affect root hair
elongation (Figure 1B).

Treatment with the fluorescent SLs and Antimycin A, an inhibitor of oxidative
ATP production and of the electron flow in the mitochondrial respiratory chain
(ATPi, 10 µM) in the agar cube resulted in a significant increase in signal in the first
two segments of the root (shoot-ward) for EGO10A-BP but not for EGO10B-BP or
the Golgi or ER, and that at least part of EGO10A-BP transport in cells is via a BFA sensitive trafficking system.

Together, the results show that EGO10A-BP is distributed in the roots mainly in the epidermal cell layer. This distribution of EGO10A-BP suggests SLs to be mainly transported in the epidermis cell layer. However it may also derived from the exogenous supplementation of EGO10A-BP molecule and the plasma-membrane orientation of the transporters in the epidermal cells. Transporters that are localized mostly to the apical plasma membrane in these cells are likely to transport the molecules mostly to apical cells, rather than to those present in adjacent cell layers.

EGO10A-BP molecules are present mostly in the cell cytoplasm. Thus, SLs may be transported sympatistically in the plant. Moreover, this transport may be dependent on the structure of the transported molecule, since EGO10A-BP or the EGO10-mD-BP molecules are transported in the root to a lesser extent (Figure 1A). Recently, SL signaling was suggested to take place in the cell nucleus (reviewed by Al-Babili and Bouwmeester, 2015). The activity of EGO10A-BP in terms of root hair elongation suggests that EGO10A-BP penetrated the nuclei at extent sufficient to acknowledge activity.

The high accumulation of EGO10A-BP in cells that were in close contact with the agar cube in the ATPi treatment, and the relatively low level of BP-signal in distant segments of the root in this treatment, suggest that once ATP-dependent processes have been disrupted, SL influx increases and its efflux decreases. Hence, it might be concluded that both the influx and efflux of EGO10A-BP are ATP-dependent, the influx negatively and the efflux positively regulated. Interestingly, auxin influx has been suggested to be gated by an active process that involves plasmodesma-localized callose deposition (Han et al., 2014). The active influx of SLs might rely on a similar gating process. Nevertheless, the absence of a significant difference in shoot-wards vs. root-ward transport of EGO10A-BP in our bioassay suggests a multidirectional flow of SLs. This may be due to the fact that only synthetic analogs were used in the experiments. Also, since EGO10-BP is a synthetic SL analog its transportation within plants may be different from that of natural SLs as the case for auxin analogs. It could also be that our results on the observed regulation of transportation may only be applicable to exogenous SLs but not to endogenous SLs.

The accumulation of SLs in BFA compartments as well as in the cytoplasm suggests that in addition to cytoplasmic diffusion, SLs are secreted via the trafficking system of the cell. Moreover, PaPDRI, the Petunia SL transporter, was shown to have a cell-type-specific asymmetric localization in different root tissues, and to be present in the plasma membrane at a polar localizations. Furthermore, treatment with BFA resulted with the accumulation of the GFP-PaPDRI signal in root cells suggesting that PaPDRI is trafficked in the cell via a BFA sensitive system (Sasse et al., 2015). Since SL transporters are delivered to the PM by BFA-sensitive trafficking, the presence of SLs in BFA bodies might be a result of this form of trafficking of their transporters. Probable involvement of active SLs trafficking in the cell and the active regulation of SL influx and efflux (see also Kretzschmar et al. 2012; Sasse et al., 2015) suggest that SL transport is a highly regulated process.


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References


Figure legends

Figure 1. (A) Signal intensity (arbitrary units) of roots treated with EGO1OA-BP, EGO1OB-BP or EGO10-mBP, with and without Antimycin A (ATPI, 10 μM). Signal was quantified (using IMAGEJ) in 9 shoot-ward segments (100 μm each) from the agar cube used to apply the fluorescent molecules to the root. All experiments were repeated at least three times, two replicates per repeat, a minimum of five seedlings per replicate in each experiment. Means of replicates were subjected to statistical analysis by Student's t-test (P ≤ 0.05). * – Statistically significant differences between means of EGO1OA-BP (blue) and EGO1OB-BP (light blue) or EGO10-mBP (green) treatments. † – Statistically significant differences between means of EGO1OA-BP and EGO10-mBP + ATPi treatments. # – Statistically significant differences between means of EGO10-mBP and EGO1OA-BP + ATPi treatments. (B) Root-hair length (μm) in root segments above the agar cubes containing EGO1OA-BP, EGO10-mBP, EGO10-mBP or naked-BP. All experiments were repeated at least three times, two replicates per repeat, a minimum of five seedlings per replicate in each experiment. Means of replicates were subjected to statistical analysis by multiple comparison Tukey-Kramer test (P ≤ 0.05). Different letters above the bars indicate statistically significant differences between means. (C) Images of roots treated with EGO10OA-BP or naked-BP. Green- EGO1OB-BP or Naked-BP signal, Blue staining- DAPI. Yellow arrows denote the epidermis cell layer. Blue, red and white arrows indicate EGO1OA-BP staining in endosomes like bodies, cytoplasm and nucleus envelop, respectively. (D) Images of roots treated with EGO10OA-BP or EGO10-mBP + ATPi followed by brefeldin A (BFA). Red – FM4-64 staining; green – EGO1OA-BP signal. BFA compartments are indicated by white arrows. Yellow arrows mark EGO1OA-BP signal in the cytoplasm. Insert: enlarged BFA body. (E) Images of roots treated with EGO10OA-BP followed by brefeldin A (BFA) treatment. In the Golgi marker-expressing lines (WT [6-1] and gal1-2) ST-RFP red – ST-RFP signal; in the endoplasmic reticulum-marker expressing line ER-cK- CFP (CS16256) red – FM4-64 staining; green – EGO1OA-BP signal; blue -ER-cK signal. BFA compartments are indicated by white arrows.
cube used to apply the fluorescent molecules to the root. (B) Signal intensity (arbitrary units) of roots treated with EGO10A-BP. Signal was quantified (using IMAGEJ) in 10 shoot-ward or root-ward segments (100 μm each) from the agar cube used to apply the fluorescent molecules to the root. All experiments were repeated at least three times, two replicates per repeat, a minimum of five seedlings per replicate in each experiment. Means of replicates were subjected to statistical analysis by multiple comparison Tukey-Kramer test ($P \leq 0.05$). Lowercase or capital letters above the bars indicate statistically significant differences between means.

**Supplemental Figure 4.** Root-hair length (μm) in root segments above the agar cubes only (not containing SL analogs) or containing EGO10A, EGO10B, or EGO10-mD, and non-treated control. Experiment consisted of four replicates per repeat, a minimum of ten seedlings per replicate. Means of replicates were subjected to statistical analysis by multiple comparison Tukey-Kramer test ($P \leq 0.05$). Different letters above the bars indicate statistically significant differences between means.

**Supplemental Figure 5.** Examples to images of roots treated with EGO10A-BP. Green - EGO10A-BP signal, Blue staining - DAPI. White arrows denote EGO10A-BP staining in nucleus.

**Supplemental Figure 6.** (A) EGO10A-BP signal in root segments that were covered with agar cubes containing EGO10A-BP or EGO10A-BP + Antimycin A (ATP1). Roots were uncut and agar cube was removed to reveal the part of the root below it. (B) Images of roots treated with EGO10A-BP and ATP1. Green - EGO10A-BP; blue - DAPI staining.

**Supplemental Figure 7.** Linear-regression formula of graph of signal intensity (arbitrary units) in roots treated with EGO10A-BP or EGO10A-BP + Antimycin A (ATP1). Signal was quantified (using IMAGEJ) in the first three shoot-ward segments (100 μm each) from the agar cube used to apply the fluorescent molecules to the root. All experiments were repeated at least three times, two replicates per repeat, a minimum of five seedlings per replicate in each experiment. Means of replicates were subjected to statistical analysis by multiple comparison Tukey-Kramer test ($P \leq 0.05$). Capital letters indicate statistically significant differences between means.
Figure 1. (A) Signal intensity (arbitrary units) of roots treated with EGO10A-BP, EGO10B-BP or EGO10A-mDP-BP, with and without Antimycin A (ATP synthetase A, 10 μM). Signal was quantified (using IMAGEJ) in 10 shoot-root segments (100 μm each) from the agar cubes used to apply the fluorescent molecules to the root. All experiments were repeated at least three times, two replicates per replicate, a minimum of five seedlings per replicate in each experiment. Means of replicates were subjected to statistical analysis by Student’s t-test (P < 0.05). * – Statistically significant differences between means of EGO10A/BP (blue) and EGO10B-BP (light blue) or EGO10A-mDP-BP (green) treatments. # – Statistically significant differences between means of EGO10A-BP and EGO10B/BP – ATP treatments. ## – Statistically significant differences between means of EGO10A-mDP-BP and EGO10-mD-BP or naked-BP. All experiments were repeated at least three times, two replicates per replicate, a minimum of five seedlings per replicate in each experiment. Means of replicates were subjected to statistical analysis by multiple comparison Tukey-Kramer test (P < 0.05). Different letters above the bars indicate statistically significant differences between means. (B) Images of roots treated with EGO10A-BP or EGO10A-mDP-BP or naked-BP. Green: EGO10A-BP or Naked-BP signal. Blue staining: DAPI. Yellow arrows denote the epidermis cell layer. Blue, red and white arrows indicate EGO10A-BP staining in endodermis like bulges, cytoplasm and nucleus envelope, respectively. (C) Images of roots treated with EGO10A-BP or EGO10A-mDP-BP – ATP followed by brefeldin A (BFA). Red – FM-64 staining; green – EGO10A-BP signal. BFA compartments are indicated by white arrows. Yellow arrows mark EGO10A-BP signal in the cytoplasm. Insert: enlarged BFA body. (D) Images of roots treated with EGO10A/BP followed by brefeldin A (BFA) treatment. In the Golgi marker expressing lines (WT [+] and pm2-2) ST-RFP red – ST-RFP signal, in the endoplasmic reticulum marker expressing lines EI-cPE (CS16256) red – FM-64 staining; green – EGO10A-BP signal; blue – cPE signal. BFA compartments are indicated by white arrows.