

**Short Communication**

**ZIFL1.1 transporter modulates polar auxin transport by stabilizing membrane abundance of multiple PINs in Arabidopsis root tip**

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## **Abstract**

Cell-to-cell directional flow of the phytohormone auxin is primarily established by polar localization of the PIN auxin transporters, a process tightly regulated at multiple levels by auxin itself. We recently reported that, in the context of strong auxin flows, activity of the vacuolar ZIFL1.1 transporter is required for fine-tuning of polar auxin transport rates in the Arabidopsis root. In particular, ZIFL1.1 function protects plasma-membrane stability of the PIN2 carrier in epidermal root tip cells under conditions normally triggering PIN2 degradation. Here, we show that ZIFL1.1 activity at the root tip also promotes PIN1 plasma-membrane abundance in central cylinder cells, thus supporting the notion that ZIFL1.1 acts as a general positive modulator of polar auxin transport in roots.

## **TEXT**

Multiple key aspects of plant development, including root patterning, growth and gravitropism, are controlled by the intercellular polarized transport of the predominant endogenous form of the phytohormone auxin, indole-3-acetic acid (IAA). While cellular auxin efflux, the rate-limiting step in this process, has been shown to rely on the coordinated action of PIN-formed (PIN) and B-type ATP binding cassette (ABCB) carriers,<sup>1-3</sup> the directionality and rate of auxin transport are mainly attributable to the highly regulated polar localization of PIN transporters.<sup>4,5</sup> Dynamic polar sorting of PINs at the plasma membrane is sustained by repeated steps of endocytic internalization and recycling back to the plasma membrane via exocytosis,<sup>6,7</sup> with this constitutive cycling controlling not only PIN subcellular localization, but also their plasma-membrane abundance and consequently their activity.<sup>8</sup> Notably, auxin appears to be the main regulator of its own asymmetric

48 distribution (reviewed in Löffke et al.<sup>9</sup>), in particular through the dual effect it exerts  
49 on PIN fate. Indeed, while short-term IAA applications inhibit the internalization step  
50 of PIN cycling promoting their stability at the plasma membrane,<sup>10,11</sup> extended IAA  
51 treatments trigger PIN protein degradation through lytic vacuolar targeting and  
52 proteasomal activity, thus reducing their plasma-membrane incidence.<sup>12-15</sup>  
53 Importantly, the combination of these auxin antagonistic effects on directional  
54 vesicular trafficking and proteasome-mediated degradation allows the positional  
55 control of PIN2 activity sustaining root gravitropism.<sup>14,15</sup>

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57 Membrane transporters from the Major Facilitator Superfamily (MFS) are single-  
58 polypeptide secondary carriers capable only of transporting small solutes in response  
59 to chemiosmotic ion gradients.<sup>16</sup> The few plant MFS carriers examined to date have  
60 been implicated in sugar, oligopeptide, nitrate and phosphate transport.<sup>17-19</sup> In  
61 addition, one *Arabidopsis thaliana* MFS member, ZIF1 (Zinc-Induced Facilitator 1),  
62 has been described as a tonoplast-localized transporter promoting zinc (Zn) tolerance  
63 by affecting vacuolar partitioning of nicotianamine, a low molecular mass chelator  
64 with high affinity for a range of transition metals.<sup>20,21</sup> Recently, we reported the  
65 functional characterization of the closest *Arabidopsis* ZIF1 homolog, the ZIFL1.1  
66 (ZIF-Like 1) transporter.<sup>22</sup> In contrast to ZIF1, our results indicate that *ZIFL1.1*  
67 expression is not regulated by the Zn external status and that the activity of the  
68 encoded carrier does not contribute to plant Zn tolerance (**Fig. 1**), as already stated by  
69 Haydon and Cobbett (2007). Instead, we found that the ZIFL1.1 transporter regulates  
70 various root auxin-related processes, such as primary root elongation upon extended  
71 challenge with the phytohormone, lateral root development and gravitropic bending.<sup>22</sup>  
72 We further showed that the proton-coupled potassium transport activity of this root

tonoplastic carrier indirectly modulates cellular auxin efflux during shootward auxin transport at the root tip. This led us to hypothesize that ZIFL1.1 function would play a role in fine-tuning polar IAA transport, particularly in situations of enhanced auxin fluxes, by regulating the activity of a specific auxin transporter. Given that ZIFL1.1 activity influences mainly if not exclusively cellular auxin efflux, a prime potential downstream target for this vacuolar MFS transporter was PIN2, to date the only polarly localized auxin efflux carrier implicated in shootward transport in root tip epidermal cells.<sup>23-25</sup> In fact, our results indicated that ZIFL1.1 adjustment of polar auxin transport rates relies at least partly on its protective effect on PIN2 plasma-membrane stability under conditions of high IAA flow that normally trigger PIN2 degradation.<sup>22</sup>

The above results did not exclude the possibility of a more general effect of the ZIFL1.1 vacuolar transporter on polar auxin efflux, namely by affecting stability and/or trafficking of other auxin efflux carriers. We therefore decided to examine the plasma-membrane distribution and abundance of PIN1 at the root tip, the rationale behind this choice stemming from our previous fluorescent protein reporter experiments using the native *ZIFL1* promoter (pro*ZIFL1*:GUS-GFP and pro*ZIFL1*:ZIFL1.1-GFP).<sup>22</sup> Indeed, at the root tip, while the ZIFL1.1 transporter is primarily expressed in the cortex and epidermis, some residual expression can also be detected in the endodermis and the central cylinder (**Fig. 2A**), indicating that the ZIFL1.1 expression domain, while predominantly coinciding with that of PIN2, also overlaps to some extent with that of PIN1.<sup>26</sup> Ectopic expression driven by the 35S promoter confers as expected high levels of the MFS protein in all root tip cell layers (**Fig. 2B**) of our *ZIFL1.1*-overexpressing lines (about 3-fold higher *ZIFL1.1* expression when

compared to the wild type; **Fig. 2C**), thus providing a setting in which the ZIFL1.1 and PIN1 carriers co-localize in the stele, pericycle and endodermis.

Immunofluorescence labeling of the native PIN1 protein at the primary root tip and subsequent visualization of the corresponding signal at the plasma membrane were performed as described previously for PIN2,<sup>22</sup> except that for quantitative analysis the whole PIN1-expressing region, i.e. endodermis, pericycle and stele,<sup>26</sup> was marked. As seen in Figure 3A, PIN1 displayed proper basal (rootward) polarization in wild-type root tip cells within its entire expression domain<sup>27</sup> and this asymmetric distribution was unaltered following prolonged IAA treatment, in agreement with previous reports<sup>13,28</sup> but unlike more short-term auxin applications that promote PIN1 lateralization in the pericycle and endodermis.<sup>29</sup> When wild-type seedlings were grown in the presence of 0.1  $\mu$ M IAA, a substantial reduction ( $P < 0.001$ , Student's *t* test) in PIN1 plasma-membrane abundance was detected, consistent with PIN1 protein degradation following prolonged IAA treatments.<sup>13,15</sup> Similar trends were observed in the *PIN2* (*eir1-4*) mutant background, except that PIN1 plasma-membrane stability appeared to be significantly enhanced particularly under IAA challenge, likely reflecting functional cross-regulation between PIN1 and PIN2 at the root tip.<sup>13,30</sup> As with PIN2,<sup>22</sup> mislocalization of the PIN1 auxin efflux carrier was not observed in either *zifl1-1* mutant or *ZIFL1.1*-overexpressing lines, regardless of whether in presence or absence of the phytohormone, indicating that ZIFL1.1 function does not interfere with PIN1 polar targeting (**Fig. 3A**). Furthermore, PIN1 incidence at the cell surface was not altered by ZIFL1.1 function under control conditions, as illustrated by the equivalent PIN1 fluorescence levels detected in wild-type, *zifl1-1* mutant and *ZIFL1.1*-overexpressing root tips. However, we found that upon prolonged IAA

123 treatment PIN1 stability at the plasma membrane was significantly decreased in the  
124 *zifl1-1* mutant (**Fig. 3**), albeit to a lesser extent than PIN2 stability.<sup>22</sup> This was not  
125 observed in the *eir1-4* mutant, indicating that the reduction in PIN1 stability induced  
126 by *ZIFL1.1* loss-of-function is not the consequence of a destabilizing effect on PIN2.  
127 By contrast, PIN1 plasma-membrane abundance was significantly enhanced by  
128 *ZIFL1.1* overexpression (**Fig. 3**). Noticeably, the magnitude of these opposite effects  
129 was more pronounced in the *ZIFL1.1*-overexpressing line than in the *zifl1-1* mutant  
130 background, in contrast to PIN2 but in agreement with the gathered gene expression  
131 data (see **Fig. 2**). Indeed, while the mild effect that *ZIFL1.1* loss-of-function exerts on  
132 PIN1 stability is in line with the low *ZIFL1.1* expression levels detected in the central  
133 cylinder of wild-type root tips (see **Fig. 2A**), the stronger effect of *ZIFL1.1*-  
134 overexpression on PIN1 plasma-membrane abundance is in accordance with the high  
135 *ZIFL1.1* expression level detected in the central cylinder when compared with the  
136 wild type (see **Fig. 2B**). Collectively, these results indicate that in addition to PIN2,  
137 and specifically in the context of a stronger polar IAA stream, activity of the *ZIFL1.1*  
138 carrier also controls the steady-state levels of PIN1 at the plasma membrane, further  
139 supporting the notion that *ZIFL1.1* acts as a positive regulator of polar auxin  
140 transport. Importantly, these findings are in agreement with *ZIFL1.1* activity  
141 modulating solely shootward auxin transport.<sup>22</sup> Indeed, while shootward IAA  
142 transport relies primarily on PIN2 shootward localization in the lateral root cap and  
143 epidermis,<sup>31</sup> Rahman et al.<sup>32</sup> demonstrated that the rootward localization of PIN2 in  
144 meristematic cortical cells is required for fine-tuning of shootward IAA transport and  
145 hence for optimal gravitropism. As for PIN1, and to the best of our knowledge, there  
146 is yet no evidence that depletion of PIN1 specifically at the root meristem would  
147 affect rootward IAA transport, firstly because the transport system that delivers auxin

to the root tip should stand above this particular region, and secondly due to the functional redundancy and compensatory properties of the PIN transport network at the root tip.<sup>13,30</sup>

The precise mechanism by which ZIFL1.1 transport activity promotes PIN stability at the plasma membrane remains to be elucidated. As ZIFL1.1 function does not appear to affect PIN2 or PIN1 polar distribution, it is tempting to speculate that ZIFL1.1 activity controls PIN steady-state levels at the plasma membrane by interfering with their vacuolar targeting and/or degradation, as already suggested for the Modulator of PIN (MOP) regulators.<sup>33</sup> Given that ZIFL1.1 influences vacuolar acidification in Arabidopsis, we hypothesize that this unexpected function of the MFS vacuolar carrier relies on its ability to generate transmembrane ionic and/or electric gradients. Noteworthy, activity of the Arabidopsis vacuolar pyrophosphatase AVP1 has been shown to promote auxin-mediated organ development by influencing apoplastic acidification through its action on the abundance and activity of the plasma membrane P-ATPase and by directly affecting PIN1 stability.<sup>34</sup> More recently, Rigas et al.<sup>35</sup> demonstrated that activity of the Arabidopsis TRH1 (Tiny Root Hair 1) transporter is similarly required for proper polar localization of the PIN1 carrier in root cells. However, in neither case was the contribution of these activities to PIN1 intracellular trafficking determined. Interestingly, both TRH1 and ZIFL1.1 exhibit potassium transport activity.<sup>16,26</sup> Potassium deficiency is known to reduce shoot growth and primary root elongation, to arrest lateral root growth and to promote alterations in auxin levels.<sup>36,37</sup> The fact that the effects of *ZIFL1.1* loss-of function and overexpression on lateral root emergence are suppressed upon cesium supply, which along with the effects of its own toxicity is perceived by root cells as a potassium

deficiency,<sup>38</sup> provides preliminary experimental evidence that the ZIFL1.1 transporter could link potassium homeostasis and auxin transport. Future experimental work should uncover the precise mode of action of ZIFL1.1 in both potassium homeostasis and polar auxin transport.

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## Figure Legends

### Figure 1

**Zinc-related phenotype of the *zifl-2* and *zifl-1* mutants and a *ZIFL1.1*-overexpressing line.**

(A) RT-PCR analysis of *ZIFL1* and *ZIF1* transcript levels in the root of 7-d old wild-type (Col-0) seedlings challenged for 48 h with various Zn supplies. Expression of the *Zinc Importer 1* (*ZIP1*) and *UBIQUITIN10* (*UBQ10*) genes is shown as plant metal status and loading controls, respectively.

(B) Effect of Zn toxicity on shoot biomass (upper panel), chlorophyll content (middle panel) and primary root elongation (lower panel) of seedlings of the wild type (Col-0), the *zifl-2* and *zifl-1* mutants, and a *ZIFL1.1*-overexpressing line (*ZIFL1.1OX*). Results are representative of two independent experiments (means  $\pm$  SD,  $n = 8$  for shoot biomass/chlorophyll content and  $n = 16$  for primary root elongation). Asterisks denote statistically significant differences from the wild type ( $P < 0.001$ ; Student's *t*-test).

### Figure 2

**Expression of the *ZIFL1.1* transporter under the control of its native or a constitutive promoter in Arabidopsis root tips.**

(A) Confocal laser scanning microscopy images of an Arabidopsis *zifl-2* mutant root tip stably expressing the *ZIFL1.1*-GFP fusion protein under the control of the endogenous *ZIFL1.1* promoter. The GFP and iodide propidium signals are visualized by green and red coloration, respectively.

(B) Confocal laser scanning microscopy images of an Arabidopsis wild-type root tip stably expressing the ZIFL1.1-YFP fusion protein under the control of the constitutive 35S promoter. Scale bars, 25  $\mu$ m.

(C) Real-time RT-PCR analysis of *ZIFL1.1* expression levels in roots of 7-d old seedlings of the wild type (Col-0) and the *ZIFL1.1OX* and *ZIFL1.1-YFPOX* overexpression lines, using *UBIQUITIN10* as a reference gene. Results are from two independent experiments, and values represent means  $\pm$  SD ( $n = 4$ ).

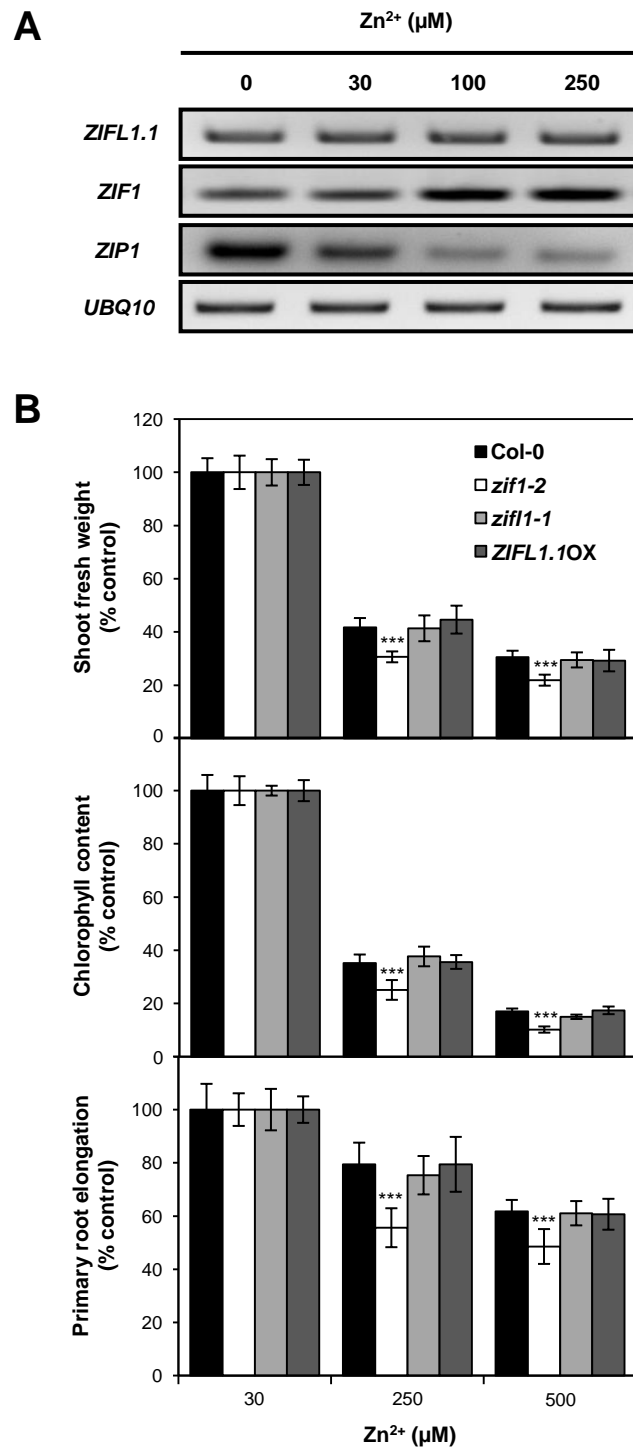
### Figure 3

#### **PIN1 immunolocalization in Arabidopsis *zifl1-1* mutant and *ZIFL1.1*-overexpressing root tips.**

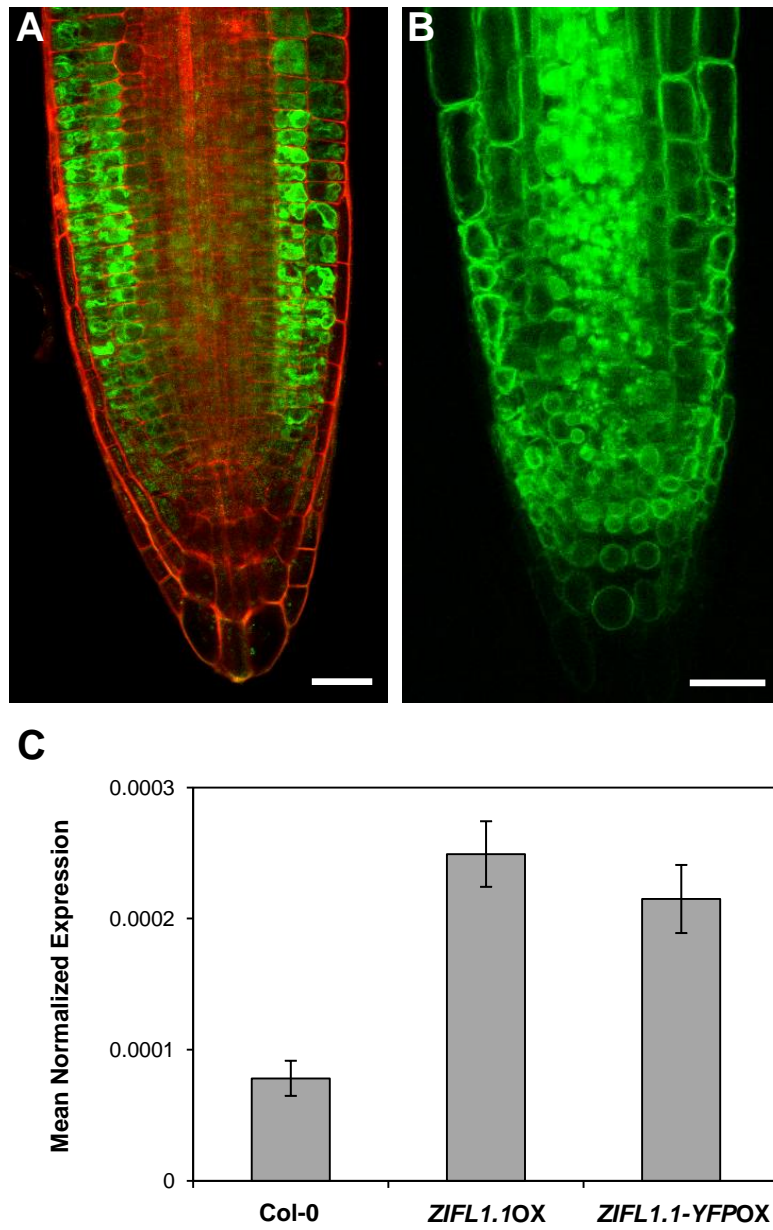
(A) Representative confocal laser scanning microscopy images of the PIN1 signal in root tips from 5-d-old wild-type (Col-0), *eir1-4* and *zifl1-1* mutant, and *ZIFL1.1*-overexpressing seedlings treated or not for 2 d with 0.1  $\mu$ M IAA. Detection settings for staining visualization were identical for all genotypes. Arrowheads indicate the polarity of PIN1 localization. e, endodermis; p, pericycle; s, stele. Signal intensities are coded blue (low) to yellow (high) corresponding to increasing intensity levels. Scale bars, 10  $\mu$ m.

(B) Quantification of the PIN1 signal at the plasma membrane of root tip cells from the stele, pericycle and endodermis in seedlings of the wild type (Col-0), the *eir1-4* and *zifl1-1* mutants, and the *ZIFL1.1OX* transgenic line. Average fluorescence (pixel) intensity values represent the mean of three independent experiments  $\pm$  SD ( $n > 23$ ). Asterisks indicate statistically significant differences from the wild type under each condition (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ; Student's  $t$  test).

**Figure 1**



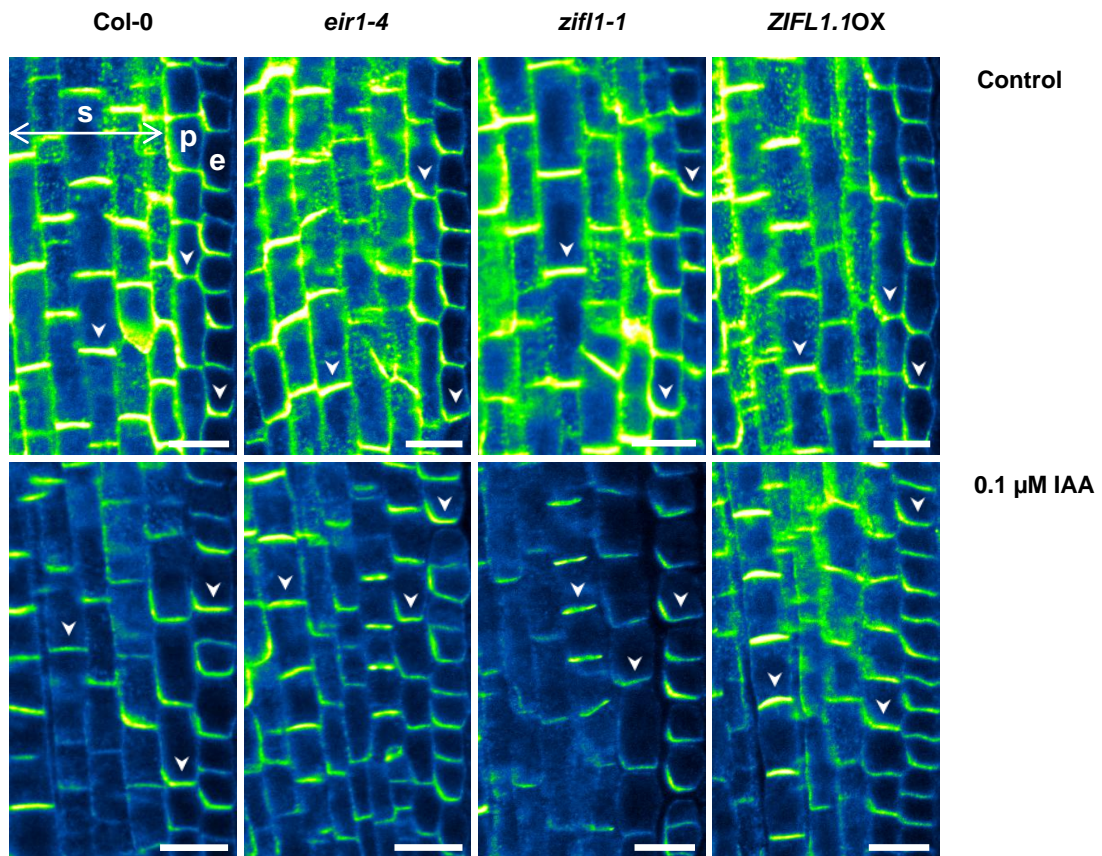
**Figure 2**





# Figure 3

**A**



**B**

