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Arabidopsis *FAB1A/B* is possibly involved in the recycling of auxin transporters

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Fab1/PIKfyve produces Phosphatidylinositol-3,5-bisphosphate(PtdIns (3,5) P_2) from Phosphatidylinositol-3-phosphate (PtdIns 3-P), and is involved not only in vacuole/lysosome homeostasis, but also in transporting various proteins to the vacuole or recycling proteins on the plasma membrane (PM) through the use of endosomes in a variety of eukaryotic cells. We previously demonstrated that Arabidopsis FAB1A/B functions as PtdIns-3,5-kinase in both Arabidopsis and fission yeast and plays a key role in vacuolar acidification and endocytosis. Although the conditional *FAB1A/B* knockdown mutant revealed an auxin-resistant phenotype to a membrane-impermeable auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), the mutant did not exhibit this phenotype to a membrane-permeable artificial auxin, naphthalene 1-acetic acid (NAA). The difference in the sensitivities to 2,4-D and NAA is similar to those of the auxin-resistant mutants such as *aux1*. Taken together, these results suggest that impairment of the function of Arabidopsis FAB1A/B might cause a defect in the membrane recycling capabilities of the auxin transporters and inhibit proper auxin transport into the cells in Arabidopsis.

Key words: auxin signaling, auxin transporter, recycling of plasma membrane proteins

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Phosphatidylinositol-3,5-bisphosphate (PtdIns (3,5) P_2) exists on the external membrane of multi-vesicular bodies (MVBs) at very low levels in eukaryotic cells,[1,2] and plays key roles in endomembrane homeostasis including endocytosis, vacuole/lysosome formation and vacuolar acidification.[1,3] PtdIns (3,5) P_2 deficiency causes an enlarged vacuolar structure in yeast and mammalian cells.[4,5] FAB1 forms a protein complex with its regulatory molecules, and synthesizes PtdIns (3,5) P_2 from PtdIns 3P.[6-9] In Arabidopsis, there are four Fab1/PIKfyve orthologs (FAB1A, FAB1B, FAB1C and FAB1D) in the genome, and the double homozygous mutant of *FAB1A* and *FAB1B* exhibited the male gametophyte lethal phenotype.[10] Previously, we reported that conditional loss-of-function and gain-of-function mutants of *FAB1A/B* impair endomembrane homeostasis and reveal various developmental phenotypes.[11] Interestingly, lateral root formation by exogenous auxin, which is known as a typical auxin-responsive phenotype, was largely impaired when *FAB1A/B* expression was conditionally downregulated or upregulated. From these results, we speculated that the defect in the endocytosis process in *fab1a/b* mutants might inhibit the precise recycling process of auxin transporters on the PM, thereby inhibiting proper auxin transport into the plant cells.[11] In this report, we tested this hypothesis to assess the sensitivity on auxin-dependent lateral root formation to a membrane permeable auxin, NAA, in the *fab1a/b* knockdown mutant.

Knockdown Mutants of *FAB1A/B* are Insensitive to 2,4-D, but Sensitive to a Membrane-Permeable Artificial Auxin, NAA

In our previous study, we showed that membrane impermeable auxin-dependent

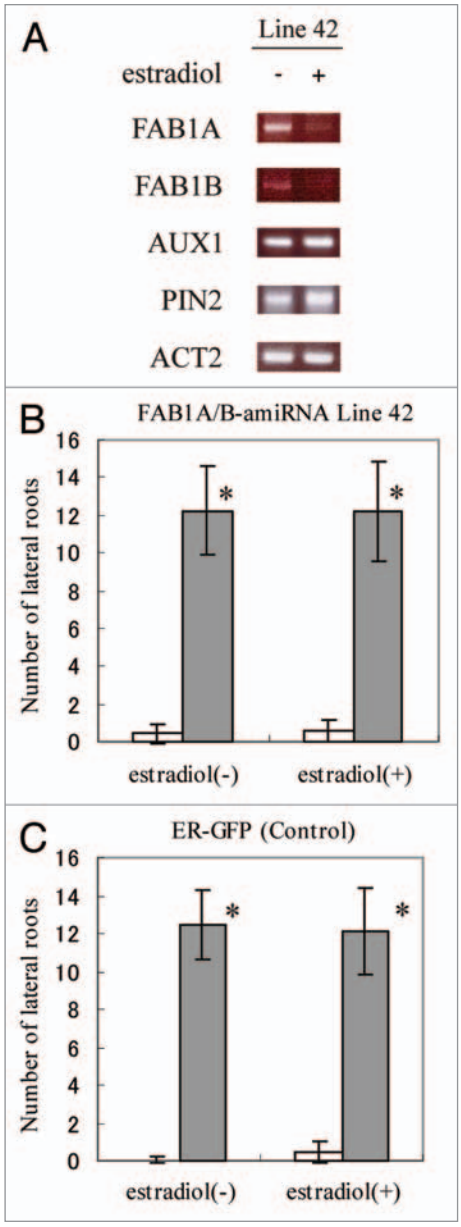

Figure 1. Downregulation of *FAB1A/B* expression does not alter the sensitivity to exogenous membrane-permeable artificial auxin, NAA, on the lateral root formation. (A) Expressions of *FAB1A*, *FAB1B*, AUX1 and PIN2 in line 42 were measured using semi-quantitative RT-PCR in the presence or absence of estradiol. The expression of *ACT2* (At3g18780) was used as an internal standard. The number of lateral roots of the amiRNA transgenic line 42, FAB1A/B-amiRNA (B) and a mock control GFP expression line, ER-GFP (C) were counted without (white bars) or with (gray bars) 0.1 μM NAA treatment in the presence of or absence of 10 μM estradiol. Bars show mean values ± standard deviations (number of lateral roots counted: FAB1A/B-amiRNA [-] estradiol, $n = 93$; FAB1A/B-amiRNA [+] estradiol, $n = 92$; ER8-GFP [-] estradiol, $n = 84$; ER8-GFP [+] estradiol, $n = 79$). Asterisks denote statistically significant differences in the number of lateral roots when compared with uninduced conditions (*$p < 0.001$; Student's t test).

lateral root formation was largely impaired when the expressions of *FAB1A* and *FAB1B* were conditionally reduced. This phenotype was reminiscent of auxin-resistant mutants such as *aux1*. AUX1 is a permease-like membrane protein, which localizes on the PM and facilitates auxin uptake into the cells. The *aux1* mutant is resistant to membrane impermeable auxins, indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), yet it is sensitive to a membrane permeable auxin, 1-naphthaleneacetic acid (NAA), indicating that AUX1 mediates the transport of auxin into the plant cells.[12-15] It has been reported that the PM and intracellular pools of AUX1 are interconnected by actin-dependent constitutive trafficking.[16] If Fab1A/B is involved in the trafficking of AUX1, a reduction in the expressions of *FAB1A/B* transcription would inhibit AUX1-mediated auxin uptake.

To test this, *FAB1A/B* knockdown plants were grown for four days on 1/2 MS agar plates with or without estradiol and then transferred to 1/2 MS plates with or without 0.1 μM NAA. After day five, the numbers of lateral roots of the mutants were counted. In the control plant and *FAB1A/B* knockdown mutant without estradiol, additional lateral root formation was induced after five days in the presence of NAA (**Fig. 1B and C**). Similarly, the lateral root formation was also induced in the presence of NAA when *FAB1A/B* expressions were downregulated (**Fig. 1A**), indicating that the sensitivity to a membrane-permeable artificial auxin, NAA, was not influenced by the *FAB1A/B* knockdown mutations (**Fig. 1B**). Note that in *FAB1A/B* knockdown plants, the expression levels of AUX1 and PIN2 did not affect the condition of the plants with or without the presence of estradiol (**Fig. 1A**), indicating that the expressions of the auxin transporters were not altered when the expression levels of *FAB1A/B* were reduced.

From these results, we concluded that the *FAB1A/B* knockdown plant is sensitive to a membrane impermeable auxin, 2,4-D, but less sensitive to a membrane impermeable auxin, NAA.

FAB1A/B Might Mediate Recycling of Auxin Transporters

FAB1A and FAB1B function as PtdIns 3P 5-kinase, FAB1/PIKfyve, on endosomes in plant as well as in yeast and mammalian cells.[1,3,11] Whitley et al.[10] reported that single homogenous mutants of *fab1a* or *fab1b* showed a curled leaf phenotype,[10] which is known as a typical phenotype of auxin-resistant mutants.[17] Furthermore, in *fab1a/b* conditional mutants, the gravitropic response of root bending and lateral root formation in response to 2,4-D

treatment was impaired. These phenotypes of *fab1a/b* conditional mutants suggest that FAB1A/B plays a role in the auxin-signaling pathway in Arabidopsis.[11] On the other hand, the *FAB1A/B* knockdown mutant completely retained the ability to respond to the membrane-permeable artificial auxin, NAA. This evidence clearly indicates that FAB1A/B do not function in the regulatory step of auxin-signaling pathway per se, but rather that they seem to function in the process of transporting auxin into the cell. An auxin efflux carrier, AUX1, transports primary natural auxins IAA and 2,4-D but not NAA.[12,13] It has been reported that constitutive trafficking and recycling of auxin transporters, AUX1 and PINs, between the endosomes and the PM led to the polar localization of these transporters.[18] In the *fab1* mutant, mislocalization of these auxin transporters may occur due to impairment of the recycling process of these proteins between the PM and intracellular pools. In future studies, direct evidence of sorting defects of these auxin transporters in the *fab1a/b* mutants should be shown in order to clarify our hypothesis.

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