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Pamela J. Hines
Science, Senior Editor

SCIENCE Manuscript 1123542

Prague, February 20, 2006

Dear Pamela Hines,

Please find attached our revised manuscript No. 1123542 "**PIN proteins perform a rate-limiting function in cellular auxin efflux**" and detailed response to reviewers.

In revising our manuscript we took into account all referees' comments and your editorial suggestions.

Please, note that in relation to changes done and addition of new data, we include one more author on the author list (Martin Kubeš). Therewith I declare that all co-authors agree with this change.

The important changes in the revised manuscript include:

1. We addressed the involvement of PGP proteins in auxin efflux.
   We conditionally expressed PGP19 in the BY-2 cells and found that PGP19 mediates auxin efflux similar to PINs. However, PGP- and PIN- mediated transport show different inhibitor sensitivity suggesting two distinct auxin efflux machineries. Furthermore, in *Arabidopsis*, PIN1 does not require function of PGP1 and PGP19 to mediate effect on plant development. This, together with absence of PGP homologues in yeast where PINs still mediate auxin efflux, strongly suggests the independence of PIN function.

2. As suggested by both reviewers, we removed the part of the phenotype data. By this, we generated a space necessary for more detailed PIN – PGP data.

3. We performed all other minor changes suggested by reviewers.

4. We heavily shortened the manuscript, so we hope that it now roughly comply with your format requirements.

Since we have included new data on PGPs, we inform you that there is a manuscript by Blakeslee et al. submitted to the Plant Cell (PLANTCELL/2006/040782) which deals with PIN-PGP interactions. However, it does not contain any data overlapping with our manuscript. We are ready to supply the whole manuscript if necessary.

We hope that we adequately addressed all the comments and suggestions.

Below please find our responses to the referees' comments.

According to your requirement we send whole manuscript including Supplementary Material by regular mail immediately.

My travel schedule for next several weeks:
I am here in Prague till the end of May, except of April 24-28, when I am at the University in Freiburg i. Br. in Germany. Anyway, all the time I will be checking my e-mail.

Thank you very much for your support.
On behalf of co-authors

Eva Zažímalová

Response to the referees' comments

Review 1

The presentation of the seedling phenotypes in PIN-transgenics is not helpful. The data do not speak to the question of whether PINs have direct roles in auxin efflux; rather raise the issue of why PIN overexpression can lead to loss-of-function phenotypes. Surprisingly, this question is not discussed, although it challenges a recently published model, according to which compensatory PIN overexpression can ameliorate pin loss-of-function defects.

We agree that PIN overexpression at the seedling level is less informative than the direct evaluation of the effects in the cell culture system. According to the suggestion, we limited this part only to demonstrate that PIN1 overexpression does not require function of PGP1 and PGP19 to exercise its effects on plant development (gravitropism, now Fig. 4).

Concerning the comment that PIN overexpression gives loss-of-function phenotypes: It is not exactly correct. It is specifically PIN1 overexpression, which causes agravitropic root growth, a hallmark of the *pin2* loss-of-function phenotype. This might be easily explained by the ectopic expression of PIN1 at the opposite side of epidermis cells than PIN2 is localized. This most likely prevents an efficient upward auxin flow in these cells. The fact that PIN1 in 35S::PIN1 plants is localized at lower side of epidermis cells (in contrast to upregulated PIN1 in *pin2* mutant – Vieten et al., 2005, Development 132 (20): 4521-4531 appears surprising. We believe that the upper PIN1 localisation in *pin2* mutant is a part of auxin-dependent compensatory mechanism, which is activated by increased auxin levels in epidermis cells of *pin2* mutant.

The study demonstrates enhanced auxin efflux, including the poorly exported auxin 2,4-D and non-functional analogs. It would be important to delimit the specificity of PIN mediated efflux by including a related compound that is not affected by PIN expression.

In the original version of the manuscript, the benzoic acid was tested – it did not compete whatsoever with ^{3}H-NAA transport. In the revised version we included the efflux (accumulation) assay with radioactively labelled tryptophan – the precursor of IAA. There was no PIN-dependent efflux activity detected.

Figures are incomplete. Despite the crucial importance of quantitative correlation throughout the paper, not even error bars are explained in many figures, let alone sample sizes and significance tests.

We entirely agree that for this type of work quantification including statistical significance must be included. We, indeed, performed this evaluation in all quantitative measurements without exception; always the standard errors or standard deviations are presented. In some cases they were so small that they are not visible because they fall into symbols (and this was mentioned in legend to Fig. 2). We apologize and where missing, the information was included.

The methods section is inadequate. As online material, this section should provide sufficient detail to enable reproduction of all results.

We agree and expanded this section.

Desirable additional points:
- dex-alone control for GVG-PINs
- correlation of PIN expression levels and auxin accumulations also in heterologous systems

The DEX control was included. Unfortunately, the PIN expression levels cannot be manipulated in yeast and HeLa cells in a controlled way.

Review 2

For example, the fact that some PINs are able to mediate auxin efflux in heterologous systems while others aren't raises additional questions as to what proteins the PINs must associate with to become functional.

The PIN1 is the only example, which was not functional in heterologous system. Two rather distant PINs – PIN2 and PIN7 were functional in both yeast and HeLas. PIN1 and PIN2 are functionally interchangeable *in planta* and PIN1 mediate auxin efflux in Arabidopsis cultured cells. We do not know why we cannot achieve a positive result with PIN1 in heterologous systems. Given the functional equivalence *in planta*, it is more probable that PIN1 cannot function in a non-plant system because of problems with stability, folding, membrane insertion or similar problem(s).

The role of the multidrug resistance proteins in auxin trafficking is not really addressed. In human cells, the PINs could be associating with PGPs. The authors claim that there is no PGP activity in yeast, but do not provide a reference or other data to support this statement.

In the revised manuscript, we addressed this issue. We overexpressed PGP19 in BY-2 cells and compared the features of PIN- and PGP-dependent auxin efflux. There are clear differences in the

inhibitor sensitivity. In addition, in Arabidopsis, PIN1 does not require function of PGP1 and PGP19 proteins to exert its effect on root gravitropism. The reference is included for no PGP activity in yeast.

Minor points:
1) The detailed description of the phenotype of the conditional gain-of-function PIN1 plants does not provide any new insight into PIN protein function. It could be placed in the supplementary information of a combined paper. The same is true for the developmental consequences of PIN overexpression on BY2 cells.

Agreed and replaced by experimental data on PGP.

2) In Figure 2, it is not clear if the number of replicates mentioned in the next to last sentence refers only to Fig. 2J or to all of the graphs in this figure.

We clarified this.