20-Sep-2019

Dr. Tzvetina Brumbarova
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Germany

RE: Localization of iron deficiency-induced transcription factor bHLH039 changes in the presence of FIT

Dear Dr. Tzvetina Brumbarova:

Thank you for giving us the opportunity to review your manuscript for publication in the Plant Physiology. The reviewers have found the manuscript of interest, but have raised a number of concerns that will require your attention. As these concerns are of some gravity and will entail substantial changes to the manuscript, you are likely to need additional time to address them. I must therefore reject the manuscript in its present form. Nonetheless, if you are able to address the points raised in review, I would welcome your resubmission of a manuscript that incorporates your responses.

In particular, I direct your attention to the following comments for consideration in a resubmitted manuscript.

The essential issue discussed by both reviewers is that the work does not examine the biological relevance of the phenomenon that has been observed. How does this finding add to our understanding of the events that lead to iron deficiency gene expression? Addressing this concern should be the focus of any subsequent submission. In addition, the reviewers pointed out several technical issues related to the design and interpretation of the experiments, and these should also be addressed wherever possible in any future submission.

Please note that any resubmission will be treated as a new entry to the system and is likely to go out for review.

If you do have any queries or concerns, you are welcome to contact me in the first instance.

Thank you again for allowing Plant Physiology to review your work. We look forward to hearing from you soon.

Sincerely,

Elsbeth L. Walker
Monitoring Editor, Plant Physiology

---------- Reviewer comments:
Reviewer #1 (Comments for the Author):

In this manuscript, the authors discovered a phenomenon that bHLH39 shuttles into nucleus in the presence of FIT. They used fluorescence to monitor the localization of bHLH39 in the presence or absence of FIT in tobacco cells, finding that bHLH39 accumulated in the nucleus when coexpressed with FIT. Further analysis confirmed that cytoplasm-nucleus ratio of bHLH39 changed in fit mutant

compared with in WT.
My comments:
1. The authors just described the phenomenon, but did not address if the shuttling of bHLH39 is required for Fe homeostasis. bHLH39 is in both cytoplasm and nucleus. Is its accumulation in nucleus really required for Fe uptake? The authors should address the biological functions of bHLH39 shuttling.

Response: At this point we can only hypothesize on the biological relevance of this phenomenon, as discussed in the manuscript. Addressing this point experimentally would require a substantial amount of time and effort and is therefore out of the scope of this manuscript.

2. They found that FIT promoted the shuttling of bHLH39, but nuclear bHLH39 was also found in the fit mutant. The conclusion that the nuclear localization of bHLH39 is dependent on FIT is improper. It is possible that other proteins can enhance nuclear accumulation of bHLH39, or bHLH39 itself can be located in the nucleus.

Response: We rechecked the text of the manuscript. We do not claim that FIT is the sole factor responsible for bHLH039 nuclear accumulation. Indeed, other proteins may play a role in this. However, our data strongly suggest that FIT has a major influence on this phenomenon. We have rephrased parts of the manuscript in order to clarify this point.

3. It is known that FIT protein is induced by -Fe. It is expected that bHLH39 protein increases in nucleus under -Fe conditions. Due to the absence of FIT protein in fit mutant, nuclear bHLH39 protein should be lower in fit than in WT. However, Figure 2E shows that nuclear bHLH39 protein level is higher in fit than in WT under -Fe conditions. Moreover, nuclear bHLH39 level is lower under -Fe conditions than under +Fe conditions in WT. It seems that FIT inhibits the nuclear accumulation of bHLH39, which is contrary to the conclusion that FIT promotes the nuclear accumulation of bHLH39.

Response: We believe that there has been a misunderstanding. The Reviewer's conclusion is based solely on the direct by-eye comparison of the signal intensities of HA-bHLH39 bands at -Fe in *fit* vs WT nuclear samples of roots (now Fig. 3E). Instead, it is necessary to take into account the difference in loading, visible in the anti-H3 immunoblot. That is why, we have normalized the HA-bHLH039 signal intensities to the respective H3 loading and used the resulting values for the cytoplasm-to-nucleus signal intensity ratio calculation. We now also present a second full set of fractionation data as a new Supplemental Figure 2, which clearly confirms our conclusions.

One potential explanation of the fact that there is a lower amount of nuclear bHLH039 protein in roots at -Fe compared to +Fe may be a similar stability control as it was already shown for the bHLH039 interaction partner FIT under this condition (Lingam et al., 2011 PlantCell; Meiser et al., 2011 PlantPhys; Sivitz et al., 2011 PlantJ). Alternatively, other factor(s) that negatively regulate(s) bHLH039 nuclear accumulation may account for the observed differential subcellular partitioning. The discussion has now been modified to better describe these hypotheses.

4. In Figure 2, the authors used cytoplasm-nucleus ratio. The ratio is based on one biological WB experiment. It is improper to draw the conclusion using only one independent experiment.

Response: As stated in the previous response to Point 3, this experiment was performed two times, yielding very comparable results. We have now included a new Supplemental Figure 2 where we present the second fractionation experiment with the corresponding quantification. Moreover, each single experiment consists of four comparisons of bHLH039 localization in *fit* versus WT, namely in

leaves and roots at + and -Fe. In each of these cases, bHLH039 shows a higher cytoplasmic to nuclear ratio in the absence of FIT than in the presence of it, in support of our conclusion.

5. In this MS, the authors observed the subcellular localization of bHLH39 in tobacco cells. Is the case similar in Arabidopsis cells? It is better to observe its localization in Arabidopsis protoplasts.

Response: We have now analyzed the subcellular localization of bHLH039 and FIT in transiently transformed Arabidopsis WT and *fit* mutant protoplasts, as suggested by the Reviewer. The data is presented as a new Figure 2. The localization results fully support the conclusions from the data obtained with tobacco leaf epidermis cells.

6. bHLH38, 39, 100 and 101 function redundantly and the bhlh39 single mutant doesn't show Fe deficiency phenotypes. Is the localization specific to bHLH39? Is also the case for other members?

Response: This question is indeed interesting but is currently not in the scope of this short report.

Reviewer #2 (Comments for the Author):

Brumbarova and colleagues describe the localization and mobility of bHLH039, and the influence of the FIT transcription factor. This research report type of manuscript is well written and relatively easy to read (I have to admit that this short format prevents the authors from providing more explanation that would sometimes help the reader). That said, it is quite descriptive and does not provide mechanistic insight into the observed phenomenon whereas there are several things that could be done to greatly improve the manuscript. Below are listed major points that limit the impact of such story and that I would like the authors to experimentally address.

Major points

1) Fig 1 and Fig 2 appear to be redundant, with Fig 1 addressing the localization of transiently overexpressed bHLH039 in tobacco leaves, and the influence of FIT co-expression while Fig 2 is based on biochemical data to evidence the same effect in stable Arabidopsis lines overexpressing bHL039 and FIT. Fig 1 should be shown as supplemental information and similar work done in Fig 1 should be done in stable lines instead (see 2) and 3) below).

Response: We cannot agree with Reviewer 2. Fig 1 is by no means redundant to Fig 2. These figures show data obtained from two different plant systems, using two different detection methods. Since we do not have stable transgenic lines expressing both fluorescently tagged bHLH39 and FIT, we have now performed transient expression of fluorescently tagged bHLH039 and FIT in Arabidopsis WT and *fit* mutant protoplasts to analyze their subcellular localization (as suggested also by Reviewer 1). Our results, which we present in a new Figure 2, fully support the conclusions of the other two experiments on the importance of FIT for bHLH039 nuclear accumulation.

To back up the observed effects of Fig 2, the authors rather need to use stable transgenic lines expressing fluorescent versions of bHLH039 and FIT. This would allow the authors to demonstrate 1) the functionality of the respective fusions by complementing respective knock outs, and 2) work in organs (i.e. roots) where FIT is actually expressed to decent levels, and 3) work at levels closer to endogenous proteins levels rather than high overexpression driven by 35S. Any other approach is subjected to many artifacts and thus limit the conclusions drawn. The evidence that bHLH039-HA

enhances Fe uptake in WT is not a solid evidence for functionality of the HA-tagged fusion protein as a non-functional bHLH39-HA may also titrate negative regulators of bHLH039 functions, this releasing the brakes on endogenous bHLH039.

Response: The functionality of the fusion proteins used in this manuscript has been demonstrated in previous publications. FIT-GFP/*fit* plants have been characterized in Gratz et al., 2019, Dev Cell. HA-bHLH039 in WT and *fit* mutant background has been analyzed in Naranjo-Arcos et al., 2017, Scientific Reports. We agree with the Reviewer that every experimental approach has its limitations. That is why we have been thorough in analyzing bHLH039 subcellular localization by currently three entirely different methods, which allows us to convincingly show the importance of FIT presence for the nuclear accumulation of bHLH039.

2) Line 81. The dual localization of FIT-GFP in tobacco is hardly visible (Fig 1A). The vast majority of the signal comes from the nucleus. Since FIT-GFP is produced in the cytosol and is highly overexpressed, the very faint signal observed cannot be taken as evidence for a dual localization. Please provide immunofluorescence to visualise the distribution of endogenous FIT in Arabidopsis root cells (using FIT antibody) and/or provide convincing evidence that a functional GFP fusion with FIT expressed under the FIT promoter is observed in the cytosol. This is required to fully convince the reader.

Response: The dual localization of FIT-GFP is not the focus of this work and has already been quantified and published in Gratz et al., 2019, DevCell and Gratz et al., 2019 NewPhytol. It is also visible in the provided images in this manuscript. Even though there is a strong nuclear FIT-GFP signal, there is also clearly a cytoplasmic portion of the signal. We would also like to point out that the use of a ß-estradiol inducible vector system for transient tobacco expression allows us to monitor subcellular localization patterns immediately after the onset of protein accumulation, reducing the possibility of overexpression artefacts.

3) To provide additional evidence that the subcellular distribution of bHLH039 observed in roots and shoots is linked to FIT being more or less present in the two organs, the authors must use FIT-OX lines. One would anticipate that bHLH039 is more nuclear-localized in FIT-OX lines. This is required to fully conclude on the dependence on FIT.

Response: It has been extensively demonstrated that FIT protein levels undergo strict post-transcriptional control (Lingam et al., 2011 PlantCell; Meiser et al., 2011 PlantPhys; Sivitz et al., 2011 PlantJ, Gratz et al., 2019 DevCell, Gratz et al., 2019 NewPhyt) and overexpression of FIT does not lead to FIT target gene activation at +Fe (see also Yuan et al., 2008 CellRes; Wang et al., 2013 Mol Plant; Gratz et al., 2019 DevCell). At +Fe, bHLH039 is not induced. Therefore, we believe that such an experiment as the one proposed by Reviewer 2 will not necessarily lead to a detectable increase in nuclear bHLH039 accumulation at +Fe, while at -Fe, the regular response is expected.

4) Fig. 2 suggests that FIT levels may account for the nuclear accumulation of bHLH039. This however appears to be independent of Fe nutrition as the ratio. Between +Fe and -Fe is conserved in the two organs tested, regardless of FIT presence. Since FIT levels vary only mildly upon changes in Fe nutrition, one may wonder what is the biological relevance of the observed effects using artificial conditions where FIT is present or absent (mutant line).

Response: The bHLH039 cytoplasm-to-nucleus ratio difference between + and -Fe is similar in 39/WT and 39/*fit* plants. However, in both cases -Fe leads to a decrease in the amount of nuclear bHLH039, meaning that Fe deficiency clearly has an effect on bHLH039 nuclear accumulation. Furthermore, lack

of FIT clearly decreases the amount of bHLH039 in the nucleus under both Fe supply conditions. This is visible when comparing the cytoplasm-to-nucleus ratios of the *fit* mutant versus the respective WT background samples in all four cases, roots and leaves, + and -Fe (in two independent biological repetitions). In each of these cases, the cytoplasm-to-nucleus ratio increases between 1.7 to 3-fold in the *fit* mutant versus wild-type background, showing that FIT has a strong effect on the nuclear localization of bHLH039.

Regarding the biological relevance, we would like to point out that even though FIT levels may vary only mildly upon changes in Fe nutrition, FIT protein activity is strongly affected by the Fe status of the plant. At the same time, bHLH039 is highly induced at -Fe (Vorwieger et al., 2007; Wang et al., 2007). Therefore, we do not claim that FIT causes an on-off bHLH039 effect but rather that the biological relevance lies in modifying the signaling strength during the plant response to Fe deficiency. We have now addressed this in the discussion.

5) The authors must address whether only active FIT interacts with bHLH39 taking advantage of mutated phosphor-dead (Ser272) and phosphor-mimic versions of FIT using biochemical and imaging approaches.

Responses: We have shown in Gratz et al., 2019 DevCell that both Ser272-phosphorylated and non-phosphorylated FIT forms can interact with bHLH039, with the non-phosphorylated S272AA FIT form showing a weaker interaction with bHLH039. We have now included this in the Discussion section. Indeed, the Reviewer's point is interesting, but out of scope of this present manuscript.

6) The authors must test if only active FIT has the ability to relocalise bHLH039 to the nucleus.

Response: The question whether active FIT is needed for bHLH039 nuclear accumulation is difficult to address because S272 phosphorylated FIT is not the only form of active FIT, as we could recently demonstrate (Gratz et al., 2019 NewPhyt). It is possible that de- and phosphorylation of FIT at several different residues modulates FIT activity. Therefore, currently it is difficult to answer this question.

7) The FRAP experiment is puzzling. No signal is observed Fig 3A, making any measures of recovery very questionable regarding whether noise is measured or actual fluorescence.

Response: The use of confocal microscopy makes it possible to distinguish real signal from noise. In the image provided in Figure 4, the nuclear bHLH039 signal is weakly visible due to the fact that the settings used for image acquisition were the same as for all other images in the figure, where oversaturation was avoided. In order to better illustrate the nuclear signal, we now provide a new Supplemental Figure 4 with enhanced signal intensity, where the nucleus (but not the nucleolus) is clearly colored by bHLH039-GFP fluorescence.

8) The cytoplasmic bHLH039-GFP foci shown Fig 3B are not observed in Fig 1, and the "plasma membrane" localization of bHLH039 shown in Fig 1 is not observed in Fig 3. How do the author explain this?

Response: The scale bar in Figure 1 is 50 µm, the one of Figure 3B (currently Figure 4B) is 5 µm. This explains why details in Figure 1 cannot be distinguished as well as in Figure 3B. We now provide a new Supplemental Figure 1 with a zoom-in on the cell periphery, demonstrating the existence of bHLH039-mCherry-containing cytoplasmic foci. The "plasma membrane" localization of bHLH039 shown in Figure 1 is also visible in Figure 3B (currently Figure 4B). The misunderstanding comes from the fact that Figure 1 represents Z-stacks from optical cross-sections of the cells, while in Figures 3B,

D, F, H (currently Figures 4B, D, F, H) we show frontal images of the periphery of the cells. Since we have realized that this was not easily understandable in the previous version of the manuscript, we have clarified this in the figure legend of the revised manuscript.

Minor points

1) Please specify the promoter driving all constructs in text and figures so this information is easily accessible to the reader.

Response: The promoter information for all used constructs has now been added to the Materials and Methods part of the manuscript and the respective figure legends.