Endocytosis of BRASSINOSTEROID INSENSITIVE1 is Partly Driven by a Canonical Tyrosine-based Motif

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2020-00384-RA 1st Editorial decision – *revision requested* **June 29, 2020**

We ask you to pay attention to the following points in preparing your revision:

1) Two of the reviewers have commented on the need to test whether the YXXΦ motif of BRI1 is also involved in vacuolar trafficking.

2) The possibility of analyzing the mutations in the YXXΦ motif to explore the mechanisms of receptor endocytosis in presence or absence of brassinolides. This would be a great addition to the manuscript but it is not essential.

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[Reviewer comments shown below along with author responses]

TPC2020-00384-RAR1 1st Revision received **Aug. 25, 2020**

Reviewer comments and **author responses**:

Reviewer #1:

This is a very nice piece of work that addresses the molecular mechanisms involved in clathrin-mediated endocytosis of the brassinosteroid (BR) receptor BRI1, which is important for its function in the perception of BRs.

The manuscript contains a large quantity of good quality data. The authors carefully analyze the contribution of several tyrosine residues in the cytoplasmic domain of BRI1, as part of putative YxxΦ canonical motifs, to BRI1 kinase activity and its AP-2 dependent clathrin-mediated endocytosis. Previously, the authors demonstrated that clathrin-mediated endocytosis of BRI1 requires the AP-2 adaptor complex. Here they convincingly show that BRI1 directly interacts with the AP2M subunit, both in vivo (BIFC) and in vitro (pull-downs), and that this interaction is mediated by the Y898KAI motif. Analysis of dynamic localization of BRI1-GFP and AP2A1-RFP together with a "departure assay" shows that BRI1 and AP-2 partially colocalize in foci and that both are co-internalized with a similar

kinetics. This is confirmed by fractionation data showing that BRI1 cofractionates with clathrin heavy chain and AP2A in the clathrin-coated vesicle fraction.

Point 1. BIFC experiments show that BRI1 interacts with other AP-2 subunits apart from AP2M, including AP2S and AP2A1, but less with AP1/2B1. The authors discuss the fact that BRI1 can bind the AP2S subunit despite the absence of di-leucine motifs (which are recognized by the AP2S subunit). However, one likely explanation for this result is that the interaction with these subunits is indirect, and that direct interaction with the AP2M subunit drives binding of the whole AP2-complex (including AP2S and AP2A1 subunits).

RESPONSE: We thank Reviewer#1 for the positive feedback. We agree that the observed interactions between BRI1 and AP2S and AP2A2 in BIFC do not suggest a direct binding. However, our conclusion is based not only on the BIFC assay but also on the performed GST pull-down experiments shown in Supplemental Figure 10 (revised manuscript) or Supplemental Figure 8 (old version).

Point 2. The plasma membrane pool of BRI1 was shown to control BR signaling, and impaired BRI1 endocytosis was shown to result in constitutive BR responses. Here the authors show that reduced endocytosis of BRI1 (Y898F) mutant causes hypersensitivity to BRs, as observed in mutants affecting CME or BRI1 ubiquitination. Surprisingly, this was not the case for other mutants that also had reduced endocytosis, which perhaps requires further discussion. Indeed, all kinase-impaired tyrosine mutants of BRI1 had a significantly reduced endocytosis, which suggests a relationship between tyrosine phosphorylation, ubiquitination and endocytosis, although the role of ubiquitin as an endocytic signal for BRI1 remains to be demonstrated.

Tyrosines 898, 961 and 1058 were shown not to be involved in kinase activity, and mutants in these residues could complement the *bri1* mutation. This means that interfering with BRI1 internalization does not cause severe phenotypic alterations. However, the authors already mention the fact that internalization of BRI1 was reduced but not abolished in the mutants. Cooperativity of tyrosine motifs in AP-2 binding (i.e., double mutants) could also be explored, as discussed, as well as the putative involvement of the T-plate complex in BRI1 endocytosis. However, the manuscript already contains a massive amount of data.

RESPONSE: We appreciate the reviewer's suggestion. We will continue to explore not only the contribution of Y961 to BRI1 internalization by generating transgenic plants expressing BRI1 containing the two Y898F and Y961F mutations simultaneously, but also the mechanisms underlying the involvement of TPLATE complex in BRI1 endocytosis in our follow-up work.

Point 3. While this manuscript reports very relevant results, it also opens interesting questions, which are nicely discussed in the manuscript and may be resolved in the future. The results of this manuscript suggest that BRI1 can undergo an AP-2 dependent basal CME and a ligand induced endocytosis dependent on BRI1 ubiquitination. This is an interesting hypothesis, which needs to be validated in the future.

In lines 326-327, the authors speculate that "the increase in the plasma membrane pool of BRI1 due to recycling cannot be ruled out". Perhaps this could be easily tested by monitoring the kinetics of relocalization of BRI1 from BFA bodies to the plasma membrane upon BFA washout.

RESPONSE: We agree with Reviewer#1 and as suggested, we performed BFA washout experiments in the presence of CHX using all BRI1 Y-to-F mutated transgenic lines. As shown in Supplemental Figure 7, by quantifying the re-localization of BRI1 from the BFA bodies to the plasma membrane, we concluded that the Y-to-F mutations in the putative YXXΦ motifs in BRI1 do not affect BRI1 recycling.

Point 4. Lines 395-396: "Notably, the ubiquitination of BRI1 in vivo was significantly reduced by the tyrosine mutations" - This is only true for the Y945F and Y956F mutations, not for the Y898F, Y961F and Y1058 mutations, which had unchanged phosphorylation.

RESPONSE: We agree with Reviewer#1 that the ubiquitination of BRI1 is significantly reduced only in the transgenic lines expressing the kinase-impaired Y945F and Y956F mutations. This is due to the fact that the ubiquitination of BRI1 depends on its activation through phosphorylation (Zhou et al., 2018, PNAS). In contrast, the Y898F, Y961F and Y1058 residues in BRI1 do not undergo phosphorylation (this manuscript) and hence do not impact the ubiquitination of BRI1. Therefore, we have adjusted the statement.

Reviewer #2:

In this manuscript, Liu and coworkers described the identification of sorting motif of BRI. This group has been working on this topic quite some time and now here they identified the sorting motif for endocytosis. To understand the molecular mechanism by which BRI1 undergoes endocytosis it is important to know the exact sorting motif recognized by the adaptors. In fact, in most cases, the sorting motif of endocytic cargoes in plants remains elusive. Authors focused on the YXXΦ motifs as candidate of sorting motif because endocytosis of BRI is dependent on the AP2. In animal cells, AP2 is known to recognize the YXXΦΦ motif. In plants, also YXXΦ motif functions as sorting motif at the TGN. Overall, it is not surprising to see that one of YXXΦ motif functions as sorting motif of BRI. However, one interesting point is that Y residue in the many YXXΦ motif can be also the target of phosphorylation. Thus, it required a large amount of work to discern the defect of phosphorylation vs. the defect of endocytosis when Y was substituted with other residues. Based on the results, authors suggested that one particular Y898XXΦ motif was the sorting motif. Thus, it represents a nice piece of work that leads to identification of the sorting motif.

Point 1. As the authors mentioned in the Discussion, the YXXΦ motif is also involved in the vacuolar trafficking from the TGN/EE. Thus, one cannot rule out the possibility that this motif may play a role in vacuolar trafficking from the TGN/EE. The blocking of vacuolar trafficking from the TGN/EE may cause recycling back to the PM from the TGN/EE. This then will give the same effect of having higher levels of BRI1 at the PM. This should be tested to claim that this motif is the sorting motif for AP2-mediated endocytosis of BRI1.

RESPONSE: We thank the Reviewer for the useful suggestion. We monitored the vacuolar accumulation of BRI1 in all transgenic lines by quantifying the vacuolar signal of BRI1-mCitrine in dark-grown plants as previously done for the $BRI1^{25KR}$ mutant (Martins et al., 2015, Nat Commun.). As shown in Supplemental Figure 8, vacuole accumulation of BRI1 quantified as relative plasma membrane BRI1-mCitrine fluorescence signal, was observed for all Y-to-F mutations, although reduced for $BRI1^{Y898F}$, $BRI1^{Y945F}$, $BRI1^{Y956F}$, and $BRI1^{Y961F}$. The reduced vacuolar accumulation of $BRI1^{Y898F}$, $BRI1^{Y945F}$, $BRI1^{Y956F}$, and $BRI1^{Y961F}$, we believe, is a result of decreased BRI1 internalization, as BRI1 recycling from the TGN/EE to plasma membrane was not affected by the mutations, as shown by the performed BFA washout experiments (Supplemental Figure 7).

Reviewer #3:

The study of Liu et al. provides novel mechanistic insights into the AP-2-dependent endocytic trafficking of the brassinosteroid receptor BRI1. This work builds on previous research of the same group back in 2013 where the authors have described the isolation of all subunits from the AP-2 complex, and have postulated the functional interaction between AP-2 and BRI1. Unlike the well-studied mammalian systems, the mechanisms of AP-mediated protein cargo sorting in plants are still elusive. In spite of the presence of canonical Tyr motifs found in the cytoplasmic domains of plant PM-localized cargo proteins, their role as endocytic signals that are recognized by AP-2 has not unambiguously been proven. Here, Liu et al. explore the functional significance of several Tyr-based motifs from the cytoplasmic domain of BRI1, and provide convincing experimental evidence that the medium subunit of AP-2 recognizes and directly binds to at least one of the studied Tyr motifs (Y898KAI), which in turn has functional implications for clathrin-mediated endocytosis of BRI1 and its signaling activity. The authors meticulously analyze the impact of the Tyr substitutions on BR signaling and the Arabidopsis growth phenotype. The studied Tyr-based motifs have been functionally classified in terms of their implication in the phosphorylation activity of BRI1. Interestingly, the growth phenotype of the complemented BRI1Y898F-mCit mutant lines does not differ from that of the wild-type BRI1-mCit in spite of the defects in BRI1 internalization and the enhanced BES1 dephosphorylation even without BL treatment. The lack of a BR-specific phenotype (e.g., narrower leaves and longer petioles) is in contrast to what has already been observed in other BRI1 mutant lines with impaired endocytosis, such as the ubiquitination-defective BRI1K25R-mCit. A possible explanation is the relatively weak BR hypersensitivity of BRI1Y898F. Overall, this study is a significant contribution to our understanding of the mechanisms of interaction of AP-2 with PM cargo proteins during CME in plant cells. The text is well structured, and most of the claims are adequately supported by the presented experimental evidence. In my opinion, the manuscript of Liu et al. definitely meets the high criteria for publication in the *Plant Cell* journal. Yet, I believe the study would benefit from a better characterization of the effect of the point mutations on BR receptor trafficking.

Point 1. As the authors have correctly pointed out in the Discussion, the elevated PM pool of BRI1 in the mutants with Y898F, Y945F, and Y956F substitutions reflects impairment of BRI1 internalization, but concomitant effects on protein recycling due to altered vacuolar targeting could also contribute to the observed phenotype. Such assumption is supported by the recent findings of Yoshinari et al. (Plant Physiol, 2019) pointing to possible interaction of Tyr-based motifs from the cytoplasmic domain of the BOR1 transporter with the medium subunits of AP-3 and AP-4 involved in vacuolar protein sorting. Most likely, visualizing the BRI1-mCitrine fluorescent signal in the vacuole after prolonged dark incubation would reveal a reduced vacuolar accumulation of mutated BRI1-mCitrine versions versus non-mutated receptor molecules (though, it would be good such a figure to be included as supplemental material). However, it would be interesting to address possible differences in BRI1 recycling by running BFA washout experiments in the presence of cycloheximide, or simply by quantifying (within each of the genotypes) the extent of decrease in the PM fluorescent signal in CHX-treated root cells after application of BFA.

RESPONSE: Please see our response to Reviewer #1 [point (3)] and to Reviewer #2 [point (2)] above.

Point 2. The Tyr mutant versions of BRI1 might be instrumental in addressing the mechanisms of receptor internalization in the presence or absence of hormonal signal. The ligand-independent internalization of BRI1 is firmly established by different research groups through much experimental evidence. However, the recent findings of Yoshinari and coworkers about the AP-2-dependent constitutive endocytosis as well as the induced AP-2-independent internalization of BOR1 raise the hypothesis for similar mechanisms of BRI1 trafficking. A good point is that Liu et al. dedicate a separate paragraph in the Discussion dealing with such a possibility. Furthermore, I would suggest analysis of the BRI1-mCit fluorescent signal at the PM and dwell time quantification in root cells of seedlings that are first grown on brassinazole to exhaust the endogenous BR levels, and then pulsed with exogenous BL (similar to the setup shown in Suppl. fig. 6). It would be interesting to compare the BL effect in the BRI1Y898F line with impaired AP-2 mediated endocytosis but with preserved kinase activity and ubiquitination versus the PM signal in the BRI1956F line where the BRI1 phosphorylation, ubiquitination, and internalization are all reduced, but the AP-2-binding Y898KAI motif is preserved.

RESPONSE: As suggested by the reviewer, we performed a BR depletion and brassinolide (BL) add-back experiment using wild type BRI1-mCitrien (mCit);bri1 line. Brassinazole (BRZ) treatment significantly increased the relative PM BRI1-mCit fluorescence intensity, and BL add-back reduced it. This result might support a ligand-dependent internalization of BRI1 along with constitutive endocytosis. However, we decided to not complicate the current manuscript with those experiments and to dedicate them to our follow-up work. Indeed using the BRI1^{Y898F} mutant might additionally support the ligand-dependency of BRI1 endocytosis.

Point 3. Lines 160-163 (Results): this paragraph deals with testing the direct interaction between the cytoplasmic domain of BRI1 and the medium subunit AP2M in vitro. Here, the results do not prove that BRI1 internalization requires a direct interaction with the AP2M, which is experimentally demonstrated later on in the manuscript. So, please rephrase the first sentence of the paragraph.

RESPONSE: We agree with Reviewer#3. The statement was rephrased as requested.

TPC2020-00384-RAR1 2nd Editorial decision – *acceptance pending* Sept. 3, 2020

We are pleased to inform you that your paper entitled "Endocytosis of BRASSINOSTEROID INSENSITIVE1 is partly driven by a canonical tyrosine-based motif" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to its presentation of scientific content, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor Sept. 16, 2020