Arabidopsis ALIX Regulates Stomatal Aperture and Turnover of ABA Receptors

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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.)

| TPC2017-00689-RA | 1st Editorial decision – declined | October 1, 2017 |

Thank you for choosing to send your manuscript entitled “Stomatal aperture and turnover of ABA receptors are regulated by Arabidopsis ALIX” for consideration at The Plant Cell. Your submission has been evaluated by members of the editorial board as well as expert reviewers in your field, and we regret to inform you that we are not able to recommend publication of this manuscript. We have not made this decision lightly. We have had input from multiple scientists, and have solicited post-review comments as well. Our present policy is to offer streamlined decisions and to not advise on the direction of the work by requesting extensive modifications or substantial additional experiments.

As you can see from the reviewers' comments they raised many important issues that should be addressed before we accept the manuscript for publication. Of course, some of them can be easily addressed by simple experiments. However, several key issues may require substantial amounts of work involving transgenic plants or a new mutant.

We would be willing to re-consider a new manuscript that fully addressed the concerns raised during this review process. If you decide to resubmit to The Plant Cell, it will be evaluated as a new submission subject to full assessment by the editorial board, and if sent for external review, a new set of reviewers is likely to be chosen. Reviewers will be asked to assess as a new manuscript (i.e. are the claims fully supported by the data; do the results presented move the field forward), and not only whether previous reviewer comments have been addressed.)

One of the editors, who was involved in the pre-review consultation (i.e. before reviewers' comments were solicited) offered the opinion that it would be important to demonstrate that the ABA-related phenotypes observed in the ALIX mutant are suppressed by removal of PYL receptors. His concern is that there are likely to be many downstream ALIX targets (especially since you mention 62 Y2H interactions with ALIX in the results). The in vivo evidence for the interactions between ALIX and PYL receptors is derived from BIFC, which in isolation is not strong demonstration that ALIX and PYLs interact at respective cellular concentrations. In the pull-downs, the IPs make use of recombinant protein, perhaps at high concentrations, which raises the question of whether the interactions would also be observed at normal cellular concentrations. These issues may be important to keep in mind as you design the experiments.
It will be insufficient to simply respond to the detailed comments of the reviewers and resubmit the manuscript. In this case it may be returned without further review. What is required are new categories of observations and/or approaches that add new understanding of mechanisms to justify the current claims made in your manuscript.

Reviewer comments:

Reviewer #1:

The manuscript by Garcia-León reports on an interaction between ABA receptors of the PYR/PYL/RCAR family and the ESCRT component ALIX. ABA receptors are soluble proteins but they are sorted for degradation at endosomes by the ESCRT machinery. Previous work from some of the authors of this manuscript showed that PYL4 interacts with FYVE1/FREE1 and VPS23A, both components of the plant ESCRT-I complex. Mutants for these ESCRT subunits showed abnormal turnover of PYL4 and altered responses to ABA. In this manuscript, a new connection between ABA receptors and the ESCRT machinery is established. PYL4 and other related ABA receptors interact with the ESCRT-III-related component ALIX. Similar to free1 and vps23a mutants, alix mutants showed abnormal ABA responses and vacuoles of abnormal morphology.

The manuscript deals with a very interesting topic since soluble cargo proteins are not usually sorted by the ESCRT machinery. I am not convinced, though, that this manuscript represents a significant contribution to what is already known in this field.

Point 1. How exactly ALIX affects PYL4 trafficking and degradation beyond the overall effect on ESCRT sorting is only superficially explored. For example, as the authors point out in the discussion, yeast ALIX/BRO1 binds ubiquitinylated cargo during ESCRT sorting and could facilitate the transferring of ubiquitinated cargo from ESCRT-I to III. However, the authors report direct interaction between ABA receptors expressed in bacteria, which are not ubiquitinylated, and ALIX. This implies that ALIX recognizes the receptor itself, not the conjugated ubiquitin moieties. This crucial difference between the known role of ALIX in eukaryotes and this potentially new role in plants is neither mentioned nor explored in the manuscript.

RESPONSE: According to our findings, Arabidopsis ALIX, as in the case of FYVE1/FREE1 and VPS23A/ELC (Belda-Palazón et al., 2016; Yu et al., 2016), directly binds PYLs. Previous reports have shown that physical binding of cargoes to components of any ESCRT complex might act as an ubiquitin-independent sorting signal at MVBs in yeast and animal systems. In this revised version of the manuscript we propose that a similar mechanism might act during ALIX-mediated sorting of PYLs; please find in the Discussion section (pages 17 and 18) the following paragraphs addressing this idea:

“Notably, however, FYVE1, VPS23A and ALIX are able to directly interact with un-modified PYL proteins (Belda-Palazón et al., 2016; Yu et al., 2016; this study). These interactions seem to be mediated by additional motifs other than their ubiquitin binding domains, which could reflect a role for FYVE1, VPS23A and ALIX in ubiquitin-independent sorting of protein cargoes. Indeed, it has been previously reported that sorting into the MVB pathway, particularly in the case of soluble proteins, can occur in the absence of cargo ubiquitination just by physical interaction of cargoes with components of any ESCRT complex (Mageswaran et al., 2014). In fact, examples for mammalian ALIX mediating ubiquitin-independent ESCRT-III/MVB sorting have been described (Dores et al., 2012, 2016), a process that might be extensive to Arabidopsis ALIX when promoting trafficking of PYLs.”

“Noteworthy, in these assays we also found interaction with PYLs through the Bro1 domain of ALIX, suggesting that different ALIX domains ensure recognition and binding of PYL proteins as an ubiquitin-independent “sorting signal” for entry into the ILVs. Accordingly, Bro1-PYL interaction was severely reduced when a Bro1 version containing the alix-1 mutation was used.”

“In this context, according to our findings, weakened interaction of ALIX-1 with PYLs should limit internalization of the latter into ILVs and their subsequent release in the vacuole lumen for degradation (Figure 8).”

Point 2. In addition, the authors quickly dismissed the contribution of the abnormally high content of ABA in alix mutant to the ABA hypersensitive phenotype they report.
RESPONSE: We agree with the Reviewer that increased ABA concentration likely contributes to ABA hypersensitivity of alix-1 mutants together with altered ABA perception and/or signalling levels. In the new version of the manuscript, both in the Results (page 9, first paragraph) and Discussion (page 18, first paragraph) we mention its potential contribution.

Point 3. To rule out that the abnormal vacuolar morphology in the alix mutant is contributing to stomatal opening, the authors treated leaves with fusicoccin, which triggers stomatal aperture. Based on the data shown in Fig 3E, the authors conclude that fusicoccin-treated stomata from alix mutant leaves showed similar degree of aperture as wild type. However, both in under dark or light conditions, Fig 3E shows a statistically significant reduction in stomatal opening in alix leaves.

RESPONSE: In this revised version, we mention this fact in page 10 as follows: “As shown in Figure 3E, FC treatments greatly recovered alix-1 defects in stomata aperture, although alix-1 stomata still displayed reduced aperture than wild-type controls upon FC treatment both under dark and light conditions.”

Then, we indicate that, as openness of FC-treated alix-1 stomata exceeded that of light-treated WT stomata (physiological conditions) we propose that alix-1 stomata are not mechanically restricted (i.e. by defects in vacuolar morphology) but, rather, constitutive signaling triggering their closure occurs in the mutant.

Point 4. Quantification of microscopy data: All colocalization and BiFC assays should be quantified. Claims like “such interaction was almost abolished when ALIX-1 version was co-infiltrated with PYL4 fusion” should be supported by quantification.

RESPONSE: According to the Reviewer’s request, we have included in this new version quantitative analyses from Z-stacking sections of agroinfiltrated leaf cells (measuring puncta per field) for all BiFC and colocalization assays. See Figures 1 and 7.

Point 5. Ideally, an independent transformation control (expression of CFP, for example), should be used in BiFC assays.

RESPONSE: As above-mentioned, rather than using an independent control we used as an internal negative control mutant ALIX-1 constructs, which displayed reduced interaction with PYL4 and VPS23A, representing therefore an optimal internal control for those assays (Figure 7B-D). Indeed, in The Plant Cell instructions for authors it is mentioned that, while using BiFC assays, “Ideally, negative controls should include a mutated version of one of the interacting proteins carrying a defect in the interaction domain or a related non-interacting protein from the same protein family” (after Kudla and Bock, 2016).

Point 6. Related to the BiFC assays, it is an excellent idea to show protein accumulation in the leaf tissues used for the BiFC analysis to demonstrate that the fusion proteins were expressed at comparable levels (supplemental Figure 3). However, this figure shows that both ALIX and PYL4 fusions were expressed at much, much higher levels when co-expressed with PYL4 and ALIX, respectively, than when coexpressed with empty vectors. This reduces the confidence on the validity of the negative controls.

RESPONSE: New Supplemental figure 4 has been prepared based on new BiFC–colocalization experiments using mCherry-ARA7 shown in Figure 7B. Again, ALIX protein versions accumulate more when PYL4 is coexpressed (which could be physiologically meaningful). However, note that, as construct combinations including the ALIX-1 mutant protein represent better controls than the empty plasmids, protein abundance comparison should be made for ALIX/PYL4 vs. ALIX-1/PYL4 samples.

Point 7. Supplemental Figure 4 shows a western blot of BiFC proteins shown in Fig 6 but without a loading control, it is impossible to know whether all these proteins were expressed at comparable levels.

RESPONSE: New supplemental Figure 5 for newly-performed BiFC assays shown in Figure 7C and D has been prepared, including appropriate loading controls.

Point 8. The authors described a yeast-two hybrid screen with two ALIX fragments that recovered 62 interactors. The list of these 62 interactors should be provided in the manuscript.

RESPONSE: Following the reviewer’s request, we have included the list of ALIX interactors as Supplemental Table 1. Note that only three proteins from the list, besides PYL9, have been reported to participate in ABA responses.
These are CAR1 (At5G37740), CAR8 (At1G23140) and VDAC3 (At5G15090). These findings are indicated in the discussion; page 18, first paragraph to open the possibility that ALIX mediates trafficking and accumulation of additional cargoes with a role in plant adaptation to ABA-related stresses.

Point 9. Only one ALIX-GFP line is shown in the manuscript? How many were analyzed? How many did show rescue of the mutant phenotypes?

RESPONSE: For the new assays performed in Figure 6, which addresses characterization of the pentuple pyl alix-1 mutant, we have used another complemented mutant line harboring a genomic fragment of the wild-type ALIX gene (gALIX/alix-1; previously published in Cardona-Lopez et al., 2015). As in the case of the GFP-ALIX line, we found complementation of the alix-1 ABA hypersensitive phenotypes in this line. The use of two different transgenic lines to complement the alix-1 mutant phenotypes strongly supports ALIX function in the control of ABA responses. This fact is mentioned in the Results section, page 13, second paragraph.

Point 10. Fig 1B, the His-PYL8 band in the pull down blot with MBP-ALIX is extremely weak. Based on this western blot, it is hard to conclude this is a positive interaction.

RESPONSE: Signal of His-PYL8 precipitated by MBP-ALIX is weak compared to that of other His-PYL fusions, particularly that of His-PYL4. However, since His-PYL8 was not precipitated by MBP alone, we consider meaningful such interaction. To indicate that coprecipitation of MBP-ALIX with different His-PYLs occurred at different rates, we have included now in the Results section; page 7, first paragraph, the following: “Interaction of ALIX with PYR/PYL receptors was further substantiated by in vitro pull downs in which recombinant His-PYLs (PYL4, 5, 8 and 9) and MBP-ALIX fusions purified from bacteria were co-precipitated upon incubation with an amylose resin, although at different rates for each receptor, showing His-PYL4 a strong interaction with ALIX (Figure 1B).

Point 11. Fig 5C, the authors show the localization of PYL4-GFP in roots stained with lysotracker red and treated with wortmannin. A control without wortmannin should be included. Much better endosomal markers than lysotracker are available. In a mutant with fragmented vacuoles like alix, it is nearly impossible to know what is an enlarged endosome and what is a small vacuole under the conditions the authors performed this experiment.

RESPONSE: We have now included a control without wortmannin, as requested, in Figure 5A. With regard to Lysotracker Red, this stain labels acidic compartments; i.e. MVBs and small vacuoles. However, the fact that LysoTracker Red-stained enlarged vesicles were visualized only upon treatment with wortmannin identifies them as endosomes instead of small vacuoles caused by the alix-1 mutation.

Point 12. The authors postulate that the alix mutant may fail to sort cargo into endosomal intralumenal vesicles (lines 332-333). That could be tested either by immunogold labeling of PYL4-GFP on endosomes or imaging of PYL4-GFP accumulation on vacuolar membranes.

RESPONSE: We did not observe localization of GFP-PYL4 on vacuolar membranes. However, we did observe GFP-PYL4 fluorescence in the outer surface of enlarged MVB in WM-treated plants supporting a defect in sorting of this protein fusion in the alix-1 mutant (Figure 5A). This notion was further supported by the fact that endogenous PYL4 protein is more abundant in the microsomal fraction of alix-1 mutants than in that of WT samples (Figure 5E).

Point 13. Figure 5B. When the authors treat seedlings with ABA, alix mutant seems to be less effective in degrading PYL4 compared to wild type controls. However, under normal growth conditions, alix mutants accumulate less PYL4 than wild type. What is the interpretation of that result?

RESPONSE: A new series of immunoblot performed using alix-1 and WT proteins extracts shows that PYL4 abundance is actually slightly increased in the alix-1 background under normal growth conditions. This difference is very significant when plants were treated with ABA, evidencing a role for ALIX in the control of PYL4 turnover. Our findings also allow us to propose the existence of an ABA-triggered negative feedback mechanism affecting PYL4 abundance. Thus, in the presence of ABA, PYL4 destabilization is more active, compared to normal growth conditions, allowing better visualization of differences in PYL4 accumulation between the alix-1 mutants and the WT.

Reviewer #2:
It has begun to emerge that the half-life of ABA receptors plays an important role in modulating ABA signaling. In this study, authors showed that ALIX, encoding an ESCRT-III-associated protein, directly interact with ABA receptors,
PYR1, PYL4, PYL8 and PYL9, targeting them for degradation in vacuole. As a result, disrupting ALIX function increased accumulation of ABA receptor in the presence of ABA, and altered ABA responsive gene expression. ALIX mutant showed increased sensitivity to ABA in seed germination, seedling establishment, seedling growth and stomatal closure. Indeed, as authors state in the manuscript that the study highlights the role of the endosome pathway in modulating ABA signaling. However, there are questions needed to be clarified.

Point 1. One possibility that cannot be excluded is that disrupting ALIX functions affects the distribution of PYLs among nucleus, cytosol and sorting endosome. The way used to extract protein and perform blot in this study may mainly reflect cytosolic accumulation of PYLs. In this case, I am afraid that the results may cause a misleading interpretation. It should be at least added that the detailed subcellular localizations of GFP-PYL4 in alix4 are as compared to the ones in wild type background.

RESPONSE: Following the reviewer’s suggestion, we analyzed the abundance of PYL4 in the cytosolic, microsomal and nuclear fractions (Figure 5E and F). Results showed that PYL4 levels are increased in the cytosol and membrane vesicles (included in the microsomal fraction) of alix-1 mutants. However, such increase in PYL4 abundance was not found in the nucleus. These results indicate that alix-1 mutation affects primarily accumulation of PYL4 in the cytoplasm (both membrane-associated and soluble in the cytosol).

Point 2. Related to point 1 above, ABA receptor PYL4 showed increase accumulation in alix mutant only in the presence of ABA, while in the absence of ABA, PYL4 levels are lower in both alix mutant and alix-GFP lines compared to wild type plants. Does this indicate ALIX-mediated PYL targeting for degradation only occur in the presence of ABA, while in the absence of ABA, ALIX homeostasis is required to maintain PYL level? A model should be proposed to discuss the role of ABA in ALIX-mediated degradation of PYLs or the possibility of ALIX-mediated redistribution of PYLs in different cellular compartments.

RESPONSE: As previously mentioned (Reviewer 1, point 13): A new series of immunoblots performed using alix-1 and WT proteins extracts shows that PYL4 abundance is actually slightly increased in the alix-1 background. This difference is very significant when plants were treated with ABA, evidencing a role for ALIX in the control of PYL4 turnover. Our findings also allow us to propose the existence of an ABA-triggered negative feedback mechanism affecting PYL4 abundance. Thus, in the presence of ABA, PYL4 destabilization is more active, compared to normal growth conditions, allowing better visualization of differences in PYL4 accumulation between the alix-1 mutants and the WT.

Discussion on the significance of different ABA-mediated mechanisms to control independently the abundance of specific PYLs at different cell compartments is included in the Discussion, page 16, second paragraph as follows:

“By modulating ABA receptor abundance at the cytoplasm, ALIX likely participates in the plant desensitization mechanisms against this hormone, repressing ABA-triggered developmental (i.e. seed germination, seedling establishment, and root growth) and water deficiency-responses (stomatal closure). The notion that trafficking of membrane-associated ABA receptors might underlie an ABA-triggered negative feedback mechanism is in line with our observation that PYL4 turnover is enhanced in response to ABA. This negative feedback mechanism may help to temporally restrict PYL4 function, allowing pulsed responses to ABA. Interestingly, ABA seems to regulate differentially the stability of specific PYLs. Thus, contrary to the case of PYL4 for which ABA prompts its destabilization, ABA treatment promotes PYL8 accumulation by inhibiting CRL4 E3 ubiquitin ligases that target it for ubiquitination and proteasomal degradation at the nucleus (Belda-Palazón et al., 2018; Irigoyen et al., 2014). Antagonistic effect of ABA on the abundance of PYLs in separate cell compartments indicates independent modulation of different responses to this hormone.”

A model to summarize our findings in this regard has been also included in new Figure 8.

Point 3. It is known that ABA responses often are different in seed germination, seedling establishment and stomata closure. alix mutant showed increased sensitivity in all cases, and look like these phenotypes are relevant to enhanced PYL accumulation in alix mutant. However, it cannot be ignored that under light stomatal apertures in alix mutant are much smaller than in wild type, implying that altered vacuolar morphology in guard cell is related to the regulation of stomatal movement. Even on the condition of light plus FC (Figure 3E), stomatal apertures in the alix mutant are still significantly smaller than wild type, further indicating that defective vacuole morphology contributes to stomatal phenotype of alix mutant.
RESPONSE: We agree with the Reviewer and comment on this fact in the new version of the manuscript (please see response to Reviewer 1, point 3). Additionally, in the discussion section (page 18, first paragraph; see below our answer to point 4), we consider the contribution of defective vacuolar morphology to defects in stomatal aperture in alix-1 leaves. Nevertheless, the analysis of stomatal aperture in pentuple pyl alix-1 mutants showed similar degree of aperture that WT controls, indicating that alix-1 stomata are not mechanically restricted and that reduced ABA signaling makes them fully operative. See Results section, page 13, last paragraph:

“Noteworthy, reduced PYL function in pent alix-1 mutants also suppressed defects in stomatal aperture found in non-treated alix-1 plants, further supporting the notion that alix-1 stomata are fully operative but highly sensitive to endogenous ABA levels.”

Point 4. Several points should be discussed, for example, the cause of the higher level of ABA and its relevancy to low PYL4 accumulation in alix mutant, as well as constitutive ABA response of alix mutant in the absence of ABA.

RESPONSE: The reason why alix-1 mutants display higher ABA levels than the WT is unknown. Such increase in ABA levels might represent a means to compensate high PYL4 abundance. Thus, increased ABA may help to reduce cytoplasmic PYL4 levels as part of the negative feedback mechanism that we describe in this study. However, as other explanations are possible, we have avoided speculation on this matter. Nevertheless, as requested by the reviewers, we include a paragraph commenting on the increased endogenous ABA levels to the ABA hypersensitive phenotypes of alix-1 mutants. This paragraph also mentions the potential contribution of altered trafficking of other ABA-related proteins, or that of defective vacuolar biogenesis during ABA-triggered closure of alix-1 stomata. See Discussion section, page 18, first paragraph:

“Such studies will help us to understand whether similar protein-protein interaction domains mediate binding of ALIX to other ABA pathway components identified in our Y2H screen, such as CAR proteins and VOLTAGE DEPENDENT ANION CHANNEL 3 (VDAC3). Though their regulatory relationships need yet to be determined, they might partially underlie the ABA hypersensitive phenotypes of alix-1 mutants. This together with defects in stomatal closure due to altered vacuolar biogenesis and increased ABA levels might also contribute to the ABA hypersensitive phenotypes of alix-1 mutants. However, the fact that reduced PYL function suppresses ABA defects in pent alix-1 plants, which invokes an effect of ALIX in the control of PYLs abundance and function, provides a simpler framework to explain alix-1 hypersensitivity to ABA.”

Point 5. Movies show the movement of BiFC signals in vesicle-like compartments in a transient assay. I feel that an endosome marker is required to show the co-localization with BiFC signals to ensure targeted PYLs in the ALIX-mediated endosome pathway.

RESPONSE: According to this request, we provide now both micrographs and videos showing colocalization of BiFC signals with the MVB marker mCherry-ARA7. Quantification analyses of colocalizing signals are also provided in Figure 1D and Figure 7B-D.

Point 6. In Figure 5A lower panel, I guess that the arrow was misplaced.

RESPONSE: This has been corrected in the new version of Figure 5A.

Point 7. In Figure 5B and C, please explain why PYL4 show different number of bands on the blot.

RESPONSE: After many attempts to elucidate the identity of those two bands, immunoblots using protein extracts from different pyr/pyl mutants showed that the upper and lower bands correspond to PYL4 (22.4 kDa) and PYR1 (21.5 kDa), respectively. The results are shown in Figure 5G. Interestingly, the effect of alix-1 mutation is less severe for PYR1 than for PYL4 (Figure 5B, C and E) which may reflect differences in their binding affinity to ALIX.

Reviewer #3:

In this manuscript, García-León et al. illustrates a molecular basis for ABA hypersensitivity in alix-1 mutants that ALIX directly binds to ABA receptors for facilitating their sorting to the vacuole for degradation, by mainly demonstrating: (1) ALIX binds to ABA receptors both in vitro and in vivo; (2) alix-1 mutants show ABA hypersensitivity during seeds germination, seedling establishment, and stomatal closure; (3) Impaired ALIX function leads to altered endosomal localization and increased accumulation of ABA receptors; (4) ALIX-1 mutant protein in alix-1 disrupted the interaction with FYVE1, VPS23A, and ABA receptors.
Overall, this work was nicely performed and data were well organized, but some of the conclusions should be further confirmed. The interesting aspect of this work is the demonstration that ALIX regulates stomata aperture as a mean to against ABA signal to control water loss through the stomata pore. However, the ESCRT-dependent vacuolar degradation of ABA receptors and the underlying regulatory mechanisms controlling the abundance or activity of ABA receptors have been reported in ESCRT components FYVE1 (Belda-Palazón et al., 2016, Plant Cell) and VPS23 (Yu et al., 2016, Mol. Plant). It is therefore not surprise that ALIX, a previously reported interacting protein of FYVE1 and VPS23, also involves in a similar regulatory mechanism. The novelty of this work is questioned in this aspect.

Point 1. Would the ABA-related phenotypes observed in the ALIX mutant be suppressed by removal of the PYL receptors?

RESPONSE: To answer this question, as above-mentioned, we generated a pentuple pyr1 pyl1 pyl4 pyl5 pyl8 mutant in the alix-1 background and analyzed different ABA-related phenotypes compared to the parental and WT controls. The pentuple mutant we used has been reported to greatly impair ABA perception and therefore responses to this phytohormone (Antoni et al., Plant Phys. 2013, 161: 931-941). As shown in Figure 6, defects in alix-1 mutant were suppressed by removal of those five ABA receptors, indicating that increased accumulation of ABA receptors largely underlies the defective ABA response caused by the alix-1 mutation.

Point 2. The authors demonstrated that both Bro1 domain and ΔBro1 (ALIX without Bro1 domain) bind to different PYLs in Y2H, and recombinant ALIX and PYLs proteins can also physically interact in pull-down assays. They concluded that "These interactions seem to be mediated by additional motifs other than their ubiquitin binding domains." However, it is necessary to further confirm in vivo whether the ALIX interacted PYL is ubiquitinated or not. To test this, the authors could purify total ubiquitylated proteins and detect the PYLs using immunoblot analysis. The authors could also check the ubiquitination level of the accumulated PYL4 in alix-1 mutant as shown in Figure 5B.

RESPONSE: Based on the current literature, we propose in this revised version of the manuscript that ALIX, and very likely ESCRT-I components FYVE and VPS23A, can mediate MVB sorting by ubiquitin-dependent and - independent recognition of cargoes. Thus, it has been reported that physical interaction of cargoes with components of any ESCRT complex can act as a “sorting signal” for cargo internalization into intraluminal vesicles of MVBs (Mageswaran et al., 2014). This mechanism also works for soluble proteins that associate to vesicles. Additional reports have shown that binding of mammalian ALIX to G protein-coupled receptors promotes their ESCRT-III/MVB sorting in an ubiquitin-independent manner (Dores et al., 2012, 2016), a process that might be extensive to Arabidopsis ALIX when promoting trafficking of PYLs. Our results showing direct binding of PYLs to ALIX support this latest scenario.

Point 3. The authors used an EMS-mutated point mutation line to study ABA hypersensitivity phenotype and related molecular studies. It would be good to use additional weak alleles (e.g. T-DNA insertion in promoter or 3'UTR) of ALIX to convince the ABA hypersensitivity phenotype.

RESPONSE: To fulfill the reviewer’s request, we followed a different, yet valid, approach by including another transgenic line in the alix-1 mutant background (gALIX/alix-1; Cardona-López et al., 2015) in our analyses (Figure 6). Characterization of this line confirmed that the ABA hypersensitive phenotypes observed in alix-1 plants are due to the partial loss of ALIX function.

Point 4. Although reconstitution of YFP fluorescence in BiFC between ALIX and PYL4 shows vesicle-like dots pattern, it would be necessary to show the co-localization of PYL4 with ALIX in transgenic Arabidopsis plant. It will be a more convincing way of demonstration the colocalization of ALIX and PYL4 in guard cells after ABA treatment.

RESPONSE: This comment is in line with that raised by the editor (point 2) for which we have answered:

Additional controls have been incorporated in the BiFC assays, together with colocalization assays of ALIX and PYL4 using the mCherry-ARA7 markers, to further support the physical interactions between ALIX and PYL receptors. Quantification analyses (counted as number of vesicles/field) on the occurrence of BiFC signal for different construct combinations, and its colocalization with MVB marker are also provided.

In these regards, we would like to highlight two facts that support ALIX-PYLs interaction in vivo:

1st Despite PYLs and ALIX localize in the cytosol and can associate with different cell compartments, physical interaction between ALIX and PYL4 occurs specifically in MVBs, which backs a functional relevance for such
Physical interactions have been tested also using an ALIX-1 mutant version which displayed reduced interaction with PYL4 and VPS23A, representing therefore an optimal internal control for those assays (Figure 7B-D). Indeed, in The Plant Cell instructions for authors it is mentioned that, while using BiFC assays, “ideally, negative controls should include a mutated version of one of the interacting proteins carrying a defect in the interaction domain or a related non-interacting protein from the same protein family” (after Kudla and Bock, 2016).

With regard to colocalization assays of ALIX and PYL4 in guard cells after ABA treatment, we generated transgenic Arabidopsis mCherry-PYL4/GFP-ALIX lines to test this. However, technical problems due to really faint signal from the mCherry-PYL4 construct and high autofluorescence from chloroplasts in guard cells, which completely masked the mCherry-PYL4 signal, precluded any colocalization assays.

Point 5. Figure 1D, the authors described “the fluorescent signal resulting from the ALIX-PYL interaction and the signal from an endocytic tracer, FM4-64, were found to colocalize in those structures”. However, the signal from FM4-64 is oversaturated dispersed signal. Moreover, coexpression with an RFP-tagged ESCRT component or endosome markers will be necessary to demonstrate the ALIX-PYL interaction in ESCRT machinery.

RESPONSE: Following the reviewers request, to demonstrate that ALIX-PYL4 interaction occurs in MVBs we included in our BiFC assays expression of the MVB marker mCherry-ARA7 (instead of performing less informative FM4-64 staining). Thus, quantification analyses (counted as number of vesicles/field) on the occurrence of BiFC signal for different construct combinations, and its colocalization with MVB marker are provided in the new version of Figure 1D.

Point 6. Both HA-PYL4 overexpressing plants and alix-1 mutant display ABA hypersensitive phenotypes. It would be more convincing to demonstrate the ABA hypersensitive phenotypes by overexpressing PYL4 in the alix-1 mutant.

RESPONSE: We are thankful to the reviewer for this suggestion. However, we believe this approach is not as informative as the previously suggested by the same reviewer; to test the effect of lack of multiple ABA receptors in the alix-1 background, which we have done in Figure 6.

Point 7. Figure 5A: As a ESCRT cargo the GFP-PYL4 is supposed to be delivered to the vacuole for degradation, vacuolar delivery of GFP-PYL4 should be impaired in alix-1 mutants.

RESPONSE: To test whether GFP-PYL4 vacuolar delivery is impaired in alix-1 mutants we analyzed the levels of GFP-PYL4 and the GFP core (a GFP-PYL4 vacuolar degradation subproduct) in WT and alix-1 mutants. As shown in Figure 5, the GFP-PYL4/GFP core ratio was much higher in alix-1 mutants than in the WT, indicating that vacuolar turnover of GFP-PYL4 is hampered in the mutants.

Point 8. Figure 5B-C. PYL4 antibody detects two close bands for endogenous PYL4 in all samples. However, after CHX treatment, this antibody only detects single PYL4 protein band. The upper band in Figure 3B probably corresponds to the modified PYL4.

RESPONSE: As mentioned in our Response to Reviewer 2, point 7; after many attempts to elucidate the identity of those two bands, immunoblots using protein extracts from different pyr/pyl mutants showed that the upper and lower bands correspond to PYL4 and PYR1, respectively. The results are shown in Figure 5G. Interestingly, the effect of alix-1 mutation is less severe for PYR1 than for PYL4 (Figure 5B, C and E) which may reflect differences in their binding affinity to ALIX.

Point 9. Discussion: In mammals, ALIX interacts with both Snf7/CHMP4 through its Bro1 domain and with TSG101 (mammalian ortholog of yeast Vps23) through a PTAP motif in the C-terminal region of ALIX (Martin-Serrano et al., 2003; Strack et al., 2003; Odorizzi, 2006). However, in both mammals and plant, it remains unknown whether ALIX binds simultaneously to both ESCRT-I and ESCRT-III complexes as bridge, or functions separately in each ESCRT complex. The statement "ALIX as a bridge between ESCRT-I and -III complexes during cargo trafficking" is weak and lack of accuracy.

RESPONSE: We agree with the reviewer that our study lacks experimental demonstration for ALIX acting as a bridge between ESCRT-I and -III complexes during cargo trafficking. Accordingly, we have reduced the strength of our claims, indicating that ALIX likely acts in mediating function of both ESCRT-I and -III activity during cargo sorting.
TPC2019-00399-RA  1st Editorial decision – revision requested  June 25, 2019

We have received reviews of your manuscript entitled "Stomatal aperture and turnover of ABA receptors are regulated by Arabidopsis ALIX." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following:

The authors should discuss in the manuscript the possibilities suggested by Reviewer #4, but we do not request at this stage extensive additional experiments. If possible also address the others issues of this Reviewer and also the comments of Reviewer #5.

Reviewer comments:

TPC2019-00399-RAR1  1st Revision received  July 2, 2019

Reviewer comments on previous submission and author responses:

Reviewer #2

This manuscript illustrates a molecular basis for ABA hypersensitivity in alix-1 mutants that ALIX directly binds to ABA receptors for facilitating their sorting to the vacuole for degradation. Overall, this work was nicely performed and data were well organized with the supplemented experiments.

Reviewer #4:

In this study, the authors investigated the function of ALIX, an ESCRT accessory protein, in the regulation of vacuolar sorting of ABA receptors PYLs and plant ABA signaling. They used comprehensively molecular and cellular approaches to demonstrate the interactions of ALIX and PYLs. By analyzing the ABA-related phenotypes of alix-1, a weak alix mutant allele with point mutation in the N-terminal BRO domain, the authors demonstrated the functions of ALIX in the regulation of vacuolar sorting and degradation of PYLs, presumably through modulating their entry into the multivesicular bodies, whose biogenesis is regulated by ESCRT machinery. Two previous studies have already demonstrated the critical functions of ESCRT components (FREE1/FYVE1 and VPS23a/ELC) in the regulation of ABA signaling by modulating the vacuolar sorting and degradation of ABA receptors PYLs. Since ALIX can be incorporated into ESCRT-I complex through association with both FREE1 and VPS23, it is not surprise to see the involvement of ALIX in ABA signaling through regulating vacuolar sorting of PYLs, a mechanism that is similar as the cases shown in the studies of FREE1 and VPS23. The overall novelty of this study is compromised.

The following concerns may help to improve the manuscript.

Point 1. Since ALIX is an important subunit of ESCRT machinery and can be incorporated into either ESCRT-I complex through association with FREE1 and VPS23 or ESCRT-III complex through association with SNF7, the alix null mutant is seedling lethal with severe defects in several cellular processes including reduced intraluminal vesicles (ILVs) inside MVBs, fragmented vacuoles and accumulation of ubiquitinated cargoes. The major function of ALIX in ESCRT-III in different organisms (yeast, mammal and plant) is to recruit the deubiquitinating enzyme AMSH3 to endosome for removing the Ub moiety before the final release of ubiquitinated cargoes into intraluminal vesicles. The exact function of ALIX in ESCRT-I remains unclear. The authors reasoned the reduced vacuolar degradation of PYLs in alix-1 mutant as because of the defect of recruitment or entry of PYLs into ESCRT-I complex. However, there may exist two other possibilities: 1) alix-1 might also have defects in the formation of ILVs inside MVBs, thereby showing a general defects in MVB-mediated vacuolar sorting of ubiquitinated cargoes, and that is why alix-1 shows diversified phenotypes (defects in phosphate homeostasis and ABA signaling); 2) alix-1 has defect in the recruitment and localization of AMSH3 to late endosome MVB, thus the ubiquitinated cargoes cannot be correctly sorted into ILVs because the Ub moiety cannot be removed from the ubiquitinated cargoes even these cargo proteins are transferred to ESCRT-III complex. The authors should test the above possibilities by performing electron microscopy analysis and AMSH3 localization study in alix-1 mutant.
RESPONSE: The possibilities suggested are indeed very feasible. Thus, impaired ILVs formation and AMSH function may also affect PYLs turnover in alix-1 mutants. Thus, we have included them in the Discussion section (Page 17, line 515 to 521) as follows: “In this context, according to our findings, weakened interaction of ALIX-1 with PYLs should limit internalization of the latter into ILVs and their subsequent release in the vacuole lumen for degradation (Figure 8). Additional effects due to impaired ALIX function, such as reduced ILV formation at MVBs and defective cargo processing by AMSH proteins (Kalinowska et al., 2015; Shen et al., 2016), cannot be disregarded as they may contribute to the inadequate vacuolar turnover of PYLs in alix-1 mutants”. With regard to providing experimental evidence about impaired ILV formation or AMSH function in the specific case of the alix-1 mutant allele, we believe it is not necessary to support the functional relevance of ALIX for ABA receptors homeostasis, which has been quite substantiated in this study. Actually, such experiments should be better included in a future study focused in understanding the biochemical and molecular consequences of the amino acid substitution found in the ALIX-1 mutant protein. This might include structural analysis of Arabidopsis ALIX-1 protein in comparison to the wild-type protein, which goes far beyond of the scope of the present study.

Point 2. The results of figure 1A show that both Bro1 and ΔBro1 are able to bind to different PYLs. Does the full-length ALIX interact with these ABA receptors in the yeast two hybrid assays? In addition, if possible, it is reasonable to test the interactions between ALIX and all the ABA receptor family proteins via a yeast two hybrid assay.

RESPONSE: Upon identifying PYL9 as an interactor of ALIX in our yeast two hybrid screen, we confirmed such interaction using full-length ALIX and both Bro1 and ΔBro1 truncated proteins (see attached “Figure 1 for reviewers” at the end of this letter). Afterwards, while testing ALIX binding to other PYR/PYL/RCAR family members, we used the Bro1 and ΔBro1 versions as they might inform about different mechanisms for protein binding to ALIX. Actually, all representative PYR/PYL/RCAR proteins, both dimeric; i.e. PYR1, and monomeric; i.e. PYL4, 8 and 9, that we tested by yeast two hybrid showed interaction with both ALIX fragments. Nevertheless, it should be noted that we used full-length ALIX protein fusions for in vivo confirmation of ALIX-PYLs interaction by bimolecular fluorescence complementation (Figure 1D and supplemental movies in the manuscript). The results clearly support that full-length ALIX is able to physically interact with all PYL protein fusions tested in planta.

Point 3. Why did the MBP also show binding to His-PYL4 as shown in Fig 1B?

RESPONSE: As the reviewer indicates, there is some non-specific binding of His-PYL4 to the resin-bound MBP in Figure 1B. However, in the same figure (right panel) it is shown that His-PYL4 binds MBP-ALIX much more efficiently, supporting a direct interaction between these two proteins (that was confirmed by additional means; Figure 1C and D).

Point 4. Does ABA treatment affect the interaction affinities between ALIX and PYLs in planta?

RESPONSE: This is an interesting question that we tested by bimolecular fluorescence complementation assays. In those assays, we did not observe any significant difference in ALIX-PYL interaction upon ABA treatment (by infiltrating Nicotiana leaves with ABA solutions at different time points and ABA concentrations) compared to mock-treated leaves expressing the same ALIX-PYL fusions. Therefore, we did not explore any further this possibility. These negative results will be included in an independent study aiming to dissect the molecular basis of the ABA-mediated negative feedback mechanism affecting PYL4 abundance, which likely involve other ESCRT machinery or vesicle trafficking components. The dissection and characterization of such a mechanism is clearly out of the scope of this study.

Point 5. Are the excess PYLs accumulated in alix-1 mutant ubiquitinated or non-ubiquitinated?

RESPONSE: According to their molecular weight, PYL4 protein bands accumulating in alix-1 plants correspond to non-ubiquitinated PYL4 (Figure 5). This notion is supported by analysis of the ubiquitination pattern of an HA-PYL4 fusion in alix-1 vs. wild-type plants. Thus, we pulled down ubiquitinated-HA-PYL4 using a commercially available resin with affinity to ubiquitin (p62 resin, Enzo Life Sciences). The results (“Figure 2 for reviewers” at the end of this letter) show similar ubiquitination rates for HA-PYL4 in alix-1 and wild-type plants, which might support a role of ALIX for targeting and sorting of unmodified PYLs as we propose in the manuscript.

Point 6. Does overexpression of ALIX promote the degradation of PYLs like the case occurred in VPS23?

RESPONSE: It would be interesting to test this hypothesis. However, unfortunately, all our efforts over the years to generate Arabidopsis plants overexpressing ALIX have been unsuccessful. Indeed, transgenic 35S:GFP-ALIX and
35S:ALIX plants obtained in our lab died shortly after germination. We also tested estradiol-inducible lines with similar results; induction of ALIX expression led to plants growth arrest and lethality even using low estradiol doses. Presumably, ALIX abundance is tightly regulated within the cell and increased ALIX function triggers a general failure in vesicle trafficking and subcellular compartmentalization. This fact precluded additional assays in this direction. (Note that we used the endogenous promoter to drive expression of ALIX in Arabidopsis transgenic lines shown in this study).

Point 7. In line 195, the reference Puga et al., 2015 is absent in the list.

RESPONSE: The references to Puga et al., 2015 have been corrected. Actually, they correspond to Puga et al., 2014, which is included in the Reference list.

Reviewer #5:
The authors addressed all the comments raised by the reviewers. I do not have any major suggestions. I think the manuscript should be accepted.

TPC2019-00399-RAR1 2nd Editorial decision – acceptance pending July 12, 2019

We are pleased to inform you that your paper entitled "Stomatal aperture and turnover of ABA receptors are regulated by Arabidopsis ALIX" 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 scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor July 26, 2019