TRAF-Family Proteins Regulate Autophagy Dynamics by Modulating AUTOPHAGY PROTEIN6 Stability in Arabidopsis

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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.)

TPC2016-00687-RA 1st Editorial decision – *declined* Oct. 5, 2016

The opposing phenotypes of the TRAF1a/b- and SINAT1/2-deficient plants do not seem to be compatible with the proposed molecular function of TRAF1a/b. Similarly, it remains unclear why TRAF1a/b loss-of-function and overexpression leads to similar effects. Different ubiquitin linkages might be the key to solving these discrepancies and data addressing this would need to be included.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2017-00056-RA Submission received Jan. 19, 2017

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

This manuscript describes a role for two TRAF family proteins in autophagy in Arabidopsis. The research is potentially interesting but incomplete, with much speculation required to interpret the results.

RESPONSE: Thank you for the expert review of our manuscript. In the revised manuscript, we have thoroughly revised the manuscript following the suggestions from the editor and reviewers. Particularly, we provided evidence to support the idea that under nutrient-rich conditions, TRAF1a/1b proteins could recruit the RING finger E3 ligases SINAT1/SINAT2 for ubiquitination and degradation of ATG6 (new Figure 8). While under nutrient-starvation conditions, TRAF1a/1b proteins are likely involved in promoting the stabilization of ATG6 by interacting with SINAT6, a SINAT protein with truncated RING finger and zinc finger domains (new Figure 8), which was shown to perform an opposing function to those of SINAT1 and SINAT2 in maintaining the ATG6 homeostasis. Consistent with this, we observed that the *sinat1 sinat2* and *sinat6* knockout mutants displayed different tolerances to nutrient deprivation and in autophagosome formation (new Figure 9). We believe these new data may properly explain the dual functions of TRAF1a/1b proteins in the regulation of autophagy dynamics in Arabidopsis. Please refer to the point-by-point responses below.

Point 1. Much of the research is performed using tagged TRAF1 fusion proteins, and these should be demonstrated to be functional by complementation of the phenotype of *traf1* double mutants.

RESPONSE: Thank you very much for this constructive suggestion. In the revised manuscript, we have now introduced the TRAF1a-FLAG fusion into the *traf1a traf1b* double mutant as suggested. As shown in new Supplemental Figure 4, we observed that TRAF1a-FLAG could completely recover the phenotypes of the *traf1a traf1b* mutant, including the dwarf growth and deficient tolerance to carbon starvation. We have included this information in Page 7 in the revised manuscript.

Point 2. The microscopy in Figure 5A is of low quality and high background. Better images are needed.

RESPONSE: As suggested, the microscopy images in new Figure 5A have now been replaced with images of better quality.

Point 3. The authors demonstrate interaction between the TRAF1s and ATG6 by yeast 2-hybrid and pull down of tagged proteins. An in vivo demonstration of interaction such as FRET or BiFC would strengthen the analysis, and also indicate the location of the interaction; does it occur on autophagosomes or elsewhere in the cell?

RESPONSE: Thank you very much for your comments and suggestions. In the revised manuscript, we used BiFC to confirm the interaction between TRAF1a and ATG6. When ATG6-nYFP and cYFP-TRAF1a were transiently co-expressed in wild-type protoplasts for 16 h under light or dark conditions, the fluorescent BiFC signals were detected in the cytoplasm under light conditions, but appeared as punctate structures under dark conditions (new Figure 6D). These findings imply that in response to starvation, TRAF1s and ATG6 proteins colocalize to autophagosome-related structures.

Point 4. The molecular function of the TRAFs in recruiting ubiquitin ligases to regulate ATG6 is an interesting hypothesis and reasonable based on the mammalian literature, but is far from complete, and the story as it stands is confusing. More information on the ubiquitination process and a more complete characterization of the *sinat1 sinat2* double mutant is necessary.

Point 4a. The *traf1* double mutant has decreased ubiquitination of ATG6 but increased ATG6 proteasomal degradation, which appears contradictory. The authors discuss the situation in animal cells in which different ubiquitin linkages have different effects on stability, either increasing or decreasing degradation. This should be tested in the *traf1* mutant to clarify the effect of ubiquitination.

RESPONSE: As shown in Figure 6, our results suggest that the *traf1a/b* double mutant had decreased ATG6 ubiquitination compare to that of wild type (new Figure 6E). Consistent with this, the proteasomal degradation of ATG6 was strongly inhibited in the *traf1a/b-1* mutant (new Figure 6F). However, we observed that in comparison to wild type, the degradation of ATG6 was also slightly reduced in the TRAF1-overexpression line TRAF1a-FLAG (new Figure 6F), suggesting TRAF1 may function as a molecular adaptor to recruit different target proteins for modulation of ATG6 stability. In the revised manuscript, we further identified SINAT6, a SINAT protein with truncated RING finger and zinc finger domains, as a negative regulator of SINAT1- and SINAT2-mediated ubiquitination and degradation of ATG6 (new Figures 7 and 8). Based on these findings, we propose that under normal growth conditions, SINAT1 and SINAT2 are key E3 ligases that regulate the interaction, ubiquitination, and degradation of ATG6, thereby suppressing autophagy. By contrast, nutrient starvation triggers the significant declines in the protein levels of SINAT1 and SINAT2 through a yet unknown mechanism, but induces the accumulation of SINAT6 protein (new Figure 8F), which directly or indirectly promotes autophagy in Arabidopsis cells.

Point 4b. The TRAFs and SINATs both lead to increased ubiquitination of ATG6, but the mutants have opposite autophagy-related phenotypes. This again could potentially be due to different Ub linkages, which should be tested. ATG6 ubiquitination and degradation should be examined in the *sinat1 sinat2* mutant for comparison with the *traf1* mutant.

RESPONSE: As stated above, we identified SINAT6 as a negative regulator of SINAT1- and SINAT2-mediated ubiquitination and destabilization of ATG6 (new Figures 7, 8 and 9) in the revised manuscript.

Moreover, we showed that compared to SINAT1 and SINAT2, SINAT6 did not have E3 ligase activity to ubiquitinate and destabilize/stabilize ATG6 in vivo (new Figures 7F and 7G). Also, the *SINAT1/2* and *SINAT6* knockout mutants displayed opposite phenotypes in tolerance to nutrient deprivation and starvation-inducible autophagosome formation (new Figure 9). Based on these findings, it is therefore conceivable that unlike the mechanism of different ubiquitin linkages to maintain the ATG6 stability in animal cells, plants use TRAF1a/b to differentially recruit the active E3 ligase SINAT1/SINAT2 or inactive SINAT6 to modulate the ATG6 homeostasis under different nutrient conditions.

Point 4c. FLAG-tagged SINAT1 or 2 should be introduced into the *sinat* double mutant to ensure complementation and to confirm that the phenotype is due to loss of SINAT.

RESPONSE: Thank you very much for the suggestion. To confirm that the phenotype in the *sinat1 sinat2* double mutant is due to loss of SINAT1 and SINAT2 proteins, we generated two knockout lines, S1/2-Cas15 and S1/2-Cas23, using the CRISPR-Cas9 system (new Supplemental Figure 10). Phenotypic analyses revealed that all three lines (*sinat1 sinat2*, S1/2-Cas15, and S1/2-Cas23) showed enhanced tolerance to carbon and nitrogen starvation to a similar level (new Figures 9A and 9B), confirming the phenotypes observed in these lines are due to the loss function of SINAT1 and SINAT2. Moreover, we performed several in vitro and in vivo assays, including protein-protein interactions, ubiquitination assays, and protein stability assays (new Figures 7 and 8), to suggest that SINAT1-FLAG, SINAT2-FLAG, and SINAT6-FLAG fusions are functional to modulate ATG6 protein stability in Arabidopsis cells.

Point 4d. The effect of the *sinat* double mutant on autophagy should be determined.

RESPONSE: As suggested, the effect of SINAT1/SINAT2 and SINAT6 knockout lines on autophagy have been determined by both confocal microscopy of eGFP-ATG8e marker (new Figures 9E and 9F) and MDC staining (new Supplemental Figure 11). Our results showed that under nutrient-rich or nutrient-starvation conditions, the numbers of autophagic puncta in the root cells of sinat1 sinat2 and sinat6 knockout lines were significantly increased and reduced, respectively, compared the eGFP-ATG8e line in wild-type background (new Figures 9E and 9F; Supplemental Figure 11). These findings provide direct evidence to support the idea that SINAT1/SINAT2 and SINAT6 play opposing role in the regulation of autophagy dynamics in Arabidopsis.

Reviewer #2:

The manuscript by Qi et al. reports the identification of TRAF-family proteins and RING finger E3 ligases that coordinately control protein levels of the core autophagy protein ATG6. By screening a large number of TRAF-family proteins, the authors identified TRAF1a and TRAF1b as autophagosome-associated proteins upon starvation. Analysis of TRAF1a/b single and double loss-of-function mutants revealed similar phenotypes as shown for autophagy-deficient mutants (e.g. hypersensitivity to nutrient limitation and stress conditions, enhanced pathogen resistance, constitutive cell death in leaves), except that the traf1a/b double mutants displayed prolonged longevity. Furthermore, TRAF1 and TRAF2 were shown to physically interact with ATG6 and to modulate its ubiquitination and turnover, which involves the activity of ATG6-binding RING finger E3 ligases SINAT1 and SINAT2. However, since TRAF1/2 proteins seem to also promote ubiquitination-dependent stabilization of ATG6, other yet unidentified E3-ligases with opposite functions than SINAT1/2 seem to be also recruited by the TRAF proteins.

Overall, this is a nicely written and interesting paper that is based on series of sound and to large extends well-performed experiments. The data provide novel insight into the molecular mechanisms underlying the regulation of the autophagy pathway in plants. TRAF protein-mediated ubiquitination of ATG proteins is well known to be involved in the regulation of autophagosome formation in mammalian cells, but it remained largely unknown whether similar mechanisms exist in plants. Therefore, the important findings of this manuscript should be of broad interest to the readers of Plant Cell. However, there are a couple of issues that should be considered before potential acceptance of the paper.

Point 1. The major concern is related to the paper by Huang et al., 2016 (Cell Host Microbe, 19, 204-215) that linked TRAF1a and TRAF1b (i.e. corresponding to MUSE13/14) to the regulation of NB-LRR immune receptor turnover.

The paper has also been touched in the discussion by Qi et al., however, it has not been sufficiently addressed in light of the finding that the *traf1a/traf1b* (*muse13 muse14*) double mutant shows an autoimmunity-phenotype. Therefore, the phenotype analysis in the double mutant should be done in comparison to the *traf1a traf1b snc1* triple mutant to uncouple the autoimmunity-related from potential autophagy deficiency-related phenotypes. This is particularly relevant for the surprising finding that *traf1a/traf1b* switches from an early senescence to an extended life span. The reason for this switch remains unknown and needs to be analysed more thoroughly.

RESPONSE: Thank you very much for this constructive suggestion. We have now included the new data (new Supplemental Figures 4C and 4D) to suggest that although the *snc1* mutation can overcome the dwarfing phenotype of *traf1a/b* (*muse13-2 muse14-1*) in the *snc1-r1 muse13-2 muse 14-1* triple mutant, it showed similar hypersensitivity to carbon starvation to that of *traf1a/b* (*muse13-2 muse14-1*), indicating that the autophagy deficiency in *traf1a/b* did not rely on the NB-LRR immune receptors. We have now included this information in Pages 7 and 8 in the Results and Page 21 in the Discussion, in the revised manuscript.

To further explore the underlying reasons of extended life span in the *traf1a/1b* mutants, we generated the TRAF1 knockdown (traf1a TRAF1b-RNAi) lines (new Supplemental Figure 5) in the revised manuscript. Phenotypic analyses revealed that, similar to that of *traf1a/b* double mutants, the traf1a TRAF1b-RNAi lines showed increased sensitivity to both carbon and nitrogen starvation in comparison with wild type (new Supplemental Figures 5A-5D). At 4 weeks old, the traf1a TRAF1b-RNAi plants were smaller in size than the wild type, but they could grow normally to mature stages (new Supplemental Figure 5E). In comparison, both traf1a TRAF1b-RNAi lines showed accelerated leaf senescence at 5 and 6 weeks old with similar life cycles to that of wild-type plants (new Supplemental Figure 5E). These findings suggest that the phenotypes of an extended life span observed in the *traf1a/b* mutants are likely due to their severe dwarfing growth. We have now included this information in Pages 8 and 9 in the revised manuscript.

Point 2. Several important conclusions in the paper are based on Western blot analysis. However, the quality of some of immunoblots is rather low and should be improved. This is in particular the case for Figure 5c (ATG8, ATG13) and 5D (ATG8) as well as Figure 6F and 6G. In addition, potential differences in the levels of ATG6 in Figure 5D and 5G in different genotypes or in response to different treatments need to be supported by at least semi-quantitative analysis. The same applies to ATG6 levels in response to different amounts of SINAT1, SINAT2, and SINAT5 in Figure 7G.

RESPONSE: Thank you very much for the suggestions. As suggested, we have now replaced the low-quality immunoblots in the original Figures 5C, 5D, 6F, and 6G (new Figures 5E, 6F and 6G) with new blots in the revised manuscript. Meanwhile, to show the differences in the levels of ATG6 and SINATs more clearly, we followed the reviewer's suggestion to include the semi-quantitative values of these immunoblots in the new Figures 6F, 6G, 7G, 8C, 8E, and 8F below each band.

Point 3. The analysis of autophagosome formation based on MDC staining and GFP-ATG8e fluorescence should be supported by quantitative analysis.

RESPONSE: As suggested, the quantitative data of autophagic puncta numbers have now been presented in new Figure 5B and 5D in the revised manuscript.

Reviewer #3:

This manuscript attempts to answer an important question of how autophagy is regulated in Arabidopsis at the level of ATG6 ubiquitination. The authors propose a mechanism whereby TRAF1a and TRAF1b stimulate SINAT1 and SINAT2 ubiquitin ligases to directly ubiquitinate ATG6 and send it for degradation. Unfortunately, the results are ambiguous and do not support the proposed mechanism. There are two major contradictions:

Point 1. Such a mechanism would imply common phenotype of TRAF1a/b- and SINAT1/2-deficient plants. However, double knockouts of TRAF and SINAT display opposing phenotypes.

RESPONSE: Thank you for the important comments. In the revised manuscript, we provided several lines of evidence to support the idea that TRAF1a/b recruit different SINAT proteins (SINAT1/2 and SINAT6) to differentially modulate the ATG6 stability under various nutrient conditions. First, both SINAT1/2 and SINAT6 physically interact with TRAF1a and ATG6 proteins in vitro and in vivo (new Figures 7A, 7B, and 8A). Second, SINAT1 and SINAT2 are directly involved in ubiquitination and degradation of ATG6, which processes are strongly abolished in the presence of SINAT6 (new Figures 8D and 8E). Third, immunoblot analyses showed that the protein levels of SINAT1/2 and SINAT6 are significantly decreased and accumulated, respectively, in response to nutrient-starvation treatment (new Figures 8F). Fourth, knockout mutants of SINAT1 and SINAT2 show enhanced tolerance to starvation and increased starvation-induced autophagosome formation, while the SINAT6 knockout mutants are deficient in tolerance to both nutrient deprivation and in starvation-induced autophagosome formation (new Figure 9). Based on these findings, we propose that under nutrient-rich conditions, TRAF1a/1b proteins could interact with the RING finger E3 ligases SINAT1/SINAT2 for ubiquitination and degradation of ATG6, while under nutrient-starvation conditions, TRAF1a/1b proteins are likely involved in promoting the stabilization of ATG6 by interacting with SINAT6, which is a positive regulator in maintaining ATG6 stability and autophagy induction. We believe these new data may properly explain the dual function of TRAF1a/1b in the regulation of ATG6 protein homeostasis and autophagy dynamics in Arabidopsis.

Point 2. Both *traf1a/b* knockout and TRAF1a overexpression abrogate ATG6 degradation.

RESPONSE: As discussed above, TRAF1a and TRAF1b play dual functions in regulating the dynamics of autophagy by facilitating SINAT1- and SINAT2-mediated proteolysis and SINAT6-mediated stabilization of ATG6. This is likely the reason why the *traf1a/b* mutant and TRAF1a-FLAG overexpression lines show abrogated ATG6 degradation. Indeed, when we measured the quantitative values for the immunoblots in Figure 6F, we observed that the proteasomal degradation of ATG6 was strongly inhibited in the *traf1a/b-1* mutant (Figure 6F). However, the degradation of ATG6 was slightly reduced in the TRAF1-overexpression line TRAF1a-FLAG in comparison to wild type (Figure 6F), suggesting that TRAF1a overexpression has a small effect on the ATG6 stability, which may be caused by the presence of SINAT6. Consistent with this notion, we observed that the TRAF1a-FLAG lines showed only small phenotypic changes in plant morphology and starvation tolerance (new Supplemental Figure 4A).

Point 3. Most of the in vivo experiments have been done using protoplasts.

RESPONSE: The reviewer is correct. For the experiments on protein interaction, ubiquitination, and protein stability, we usually needed to co-express two or three fusion proteins to test their reciprocal effects or interactions (new Figures 6, 7, and 8). Sometimes, the co-expression analysis had to be performed in both wild type and *traf1a/b* double mutant backgrounds (new Figures 8B and 8C). It is therefore more convenient to use the protoplast system, which has the advantages in dealing with such complex assays and has frequently been used by biologists in relevant areas (Suttangkakul et al., 2012 Plant Cell 23: 3761; Zhuang et al., 2013 Plant Cell 25: 4596; Wang et al., 2015 Plant Cell 27: 3128). We have also noted the shortcomings of the protoplast system; therefore, we have tried our best to reduce its influence on the experimental results. For example, we biologically repeated all of the experiments at least three times to ensure the stability and reliability of the obtained data. Moreover, using the stable transgenic lines, we tested the ultimate effects of ubiquitination on TRAF1 and ATG6 proteins (new Figure 6G), or nutrient starvation on the SINAT1, SINAT2, and SINAT6 proteins (new Figure 8F) by western blot analyses. Finally, our comprehensive genetic analyses covered all of the proteins analyzed in the protoplasts and showed consistent phenotypic changes in comparison with the in vivo biochemical assays, further confirming the reliability of data observed from the protoplasts.

Point 4. Data showing interaction/a lack of interaction between TRAF1a/b and SINAT1/2 are missing.

RESPONSE: Thank you very much for this important suggestion. Although we lack direct evidence to show an interaction/lack of interaction between TRAF1a/b and SINAT1/2, we provided new data in the revised manuscript to show that upon carbon starvation, the protein levels of SINAT1 and SINAT2

declined significantly in a time-dependent manner from 6 to 24 h after treatment (new Figure 8F). By contrast, the SINAT6 protein accumulated simultaneously at 24 h after starvation (new Figure 8F). These findings strengthen the hypothesis that TRAF1a and TRAF1b function as molecular adaptors that are essential for facilitating the differential availability of SINAT1/SINAT2 and SINAT6 on modulating ATG6 protein under various growth conditions. We have now included this information on Pages 16, 17, and 21 in the revised manuscript.

Point 5. Formation of a protein complexes) that is(are) proposed to control autophagy should be investigated in vivo (using co-IP and/or FRET) under conditions inducing and suppressing autophagy.

RESPONSE: Thank you very much for your comments and suggestions. In the revised manuscript, we used BiFC to confirm the interaction between TRAF1s and ATG6. When ATG6-nYFP and cYFP-TRAF1a were transiently co-expressed in wild-type protoplasts for 16 h under light or dark conditions, the fluorescent BiFC signals were detected in the cytoplasm under light conditions, but were observed as punctate structures under dark conditions (Figure 6D). These findings imply that in response to autophagy induction, TRAF1s and ATG6 proteins colocalize to the autophagosome-related structures.

Point 6. MDC is not a specific marker of autophagosomes.

RESPONSE: Thank you for the comment. We followed previous publications (Contento et al., 2005 Plant J. 42: 598; Chen et al., 2015 Autophagy 11: 2233) to perform the MDC staining analysis, which is deemed as an effective tool to label autophagosome in Arabidopsis cells. Moreover, to ensure that the MDC-labeled punctate structures are autophagosomes (Figure 5A and Supplemental 11A), we validated the findings of MDC staining by using a stable transgenic line expressing eGFP-ATG8e fusion (Xiao et al., 2011 Plant Cell 22: 1463) as an autophagosome marker (Figures 5C and 9E).

Point 7. How do authors know for sure that low mobility species on the anti-ATG6-HA western blots are ubiquitinated ATG6?

RESPONSE: Good point. To ensure the low mobility species on the anti-ATG6-HA immunoblots are ubiquitinated ATG6, we probed the blots with both anti-HA and anti-Ub antibodies. In both cases, the *traf1a/b* mutant showed clearly reduced intensity in the low mobility bands in comparison with wild type (Figure 6E).

Point 8. Analysis of autophagy markers in SINAT1/2-deficient plants is missing. It is still unclear whether SINAT1/2 are required for the progression of autophagy.

RESPONSE: As suggested, the effects of SINAT1/SINAT2 and SINAT6 knockout lines on autophagy have been determined by both confocal microscopy of eGFP-ATG8e marker (new Figures 9E and 9F) and MDC staining (new Supplemental Figure 11). Our results showed that under nutrient-rich or nutrient-starvation conditions, the numbers of autophagic puncta in the root cells of *sinat1 sinat2* and *sinat6* knockout lines significantly increased and decreased, respectively, compared with the eGFP-ATG8e line in the wild-type background (new Figures 9E and 9F; Supplemental Figure 11). These findings suggest that SINAT1/SINAT2 and SINAT6 play opposing role in the regulation of autophagy dynamics in Arabidopsis.

TPC2017-00056-RA 1st Editorial decision – *revision requested* **Feb. 8, 2017**

Your ms shows that TRAF1A/B proteins regulate autophagy by modulating the stability of the ATG6 protein by acting as adaptors to recruit E3 ligases. This represents an interesting and novel mechanism in the regulation of autophagy, however both reviewers have made suggestions how you could further strengthen and streamline your ms.

In particular, it will be important to integrate published findings on TRAF1A/B or MUSE13/14, respectively, and to provide all the requested controls so that all conclusions made are indeed justified.

---------- Reviewer comments:

[Provided below along with author responses]

TPC2017-00056-RAR1 1st Revision received Feb. 27, 2017

Reviewer comments on previous submission and **author responses**:

Reviewer #1

In plants, we know very little about the regulation of autophagy. In this manuscript, the authors have discovered that TRAF1A/B proteins regulate autophagy by modulating the stability of ATG6 protein. They further show that TRAF1A/B functions as adaptors to recruit E3 ligases that affect stability of ATG6, thereby autophagy induction. I think they provide compelling evidence on a novel regulatory pathway. Most of the data are presented with quantifications, which makes it a lot more convincing. Although the manuscript is very data intense, the authors explain their stories in a clear manner.

Their results could be summarized in three points:

1. *traf1a/b* mutants phenocopy autophagy mutants.

2. TRAF1A/B associate with ATG6 and regulate its turnover

3. To regulate ATG6 turnover, TRAF1A/B recruit E3 ligases SINAT1/2 and SINAT6. SINAT1/2 reduce ATG6 levels, whereas SINAT6 compete with SINAT1/2 and enhance ATG8 protein levels.

Since *traf1a/b* mutants have been characterized in a recently published paper, I recommend shortening the sections on genetic characterization of the mutants. After addressing the issues outlined below, I believe this story will be of great interest for the readers of The Plant Cell.

Genetic characterization of the mutants is nicely done. However the authors need to significantly improve the part where they demonstrate complex formation between TRAF1A/B, ATG6 and E3 ligases.

Point 1. In general for the WB experiments, the blots are cropped too much. The authors should leave some space on each side of the bands. Inputs are missing and even after the IP, the protein levels are not equal. In some cases, they have played with the brightness/contrast of the blots too much (for example Fig8C). They have nicely quantified band intensities in their WB experiments. However it is not very clear how they did this. To me most of the bands seem to be saturated already. They should include a section on this in the materials and methods. As loading controls in some cases they used transfected GFP, whereas in other cases they used total protein measurements. They should be consistent throughout the paper and use GFP as control for the protoplast experiments.

RESPONSE: Thank you for careful review of the figures. We have now improved all of the blots as suggested. As suggested, we have now included the input for both tags in Figures 6C, 7B, and 8A in the revised manuscript. Particularly, we repeated the CoIP assay for Figure 7B, and tried our best to ensure that equal protein amounts were loaded in each lane. Our new data clearly show that SINAT1, SINAT2, SINAT5-S1, and SINAT6, but not SINAT3 and SINAT4, interact with ATG6. We have now used the original blots to avoid adjustment of brightness/contrast too much in these Figures. As suggested, we have included the method we used to quantify the band intensities of blots in the Materials and Methods in the revised manuscript (Page 28). Indeed, in some of the protoplast experiments, we coexpressed the GFP vector to monitor the transformation efficiency (Original Figures 6E and 6F), while for all of the blots, we used Ponceau S to stain the membranes before western blot analyses to make sure of the successful transfer of proteins from the gels to the membranes. As suggested by both reviewers, we therefore used the Coomassie-stained gels with the Ponceau-staining membranes as loading controls throughout the revised manuscript, which should directly reflect the exact amounts of proteins reacted with the antibodies and were previously used as loading controls for western blot analyses (Hackenberg et al., 2013 Plant Cell 25: 4616; Munch et al., 2015 Plant Cell 27: 463; Nhuiyan et al., 2016 Plant Cell 28: 3020).

Point 2. As the authors mention in the introduction, autophagy plays a positive role in life-span by clearing out damaged organelles and protein complexes. In Fig3C, unlike the autophagy mutants, the authors claim that *traf1a/b* double mutants have an extended life span. The *traf1a/b* mutant is dwarf as shown in Fig3A, so claiming they have extended life span is not accurate. The whole developmental program is significantly delayed in the *traf1a/b* mutant compared to WT plants, so they can't compare this mutant to WT plants. I think that data should be removed.

RESPONSE: We agree with the reviewer's comment. This comparison was suggested by a reviewer in the first round of review and we thought it was necessary to measure the lifespan of *traf1a/b* mutants although they show a dwarf phenotype. In the revised manuscript, we therefore think it is better to retain Figure 3C by moving this result into Supplemental Data (see new Supplemental Figure 5).

Point 3. Line297-299. "deletion of TRAF1a and TRAF1b resulted in increased and decreased resistances to bacterial and fungal pathogens, respectively." the authors only tested one bacteria and one fungal species, they can't generalize like this. *traf1a-b* double mutant most probably will be more susceptible to a biotrophic pathogen such as powdery mildew. The phenotype they have observed is life style dependent as shown previously by Shirasu and Nurnberger labs.

RESPONSE: Good point. In the revised manuscript, we have modified the sentence to "deletion of TRAF1a and TRAF1b resulted in increased and decreased resistances to P. st. DC3000 and B. cinerea, respectively." We also thank the reviewer very much for the important suggestion for future studies of the susceptibility of *traf1a/b* double mutant to biotrophic pathogens such as powdery mildew.

Point 4.In Figure 5A-D, using confocal microscopy, they show *traf1/b* mutant have defects in autophagosome formation. In Fig5E, using WBs they suggest that *traf1a/b* double mutant have higher levels of ATG8 proteins and they claim this is an indication of a defect in the autophagy pathway. This figure has several issues:

a) In the WT, in response to C and N starvation, we should see changes in ATG protein levels. Previously Vierstra lab has shown that ATG1 and ATG13 levels rapidly decrease under starvation conditions (Suttangkakul, 2011, Plant Cell). We don't see this in these WBs.

b) Also for ATG8, the lower band is commonly used as a read-out for measuring autophagic flux. C and N starvation conditions show different patterns for this band. The authors should either discuss this or repeat these experiments.

RESPONSE: We agree with the reviewer's comment that the protein levels of ATG1a and ATG13a should decrease under starvation conditions (Suttangkakul, 2011, Plant Cell 23: 3761). As reported by Suttangkakul et al., (2011), the abundances of ATG1a and ATG13a were associated with the degree of starvation induced by nutrient deprivation. In particular, when the plants were treated with both carbon and nitrogen starvation, the ATG1a/13a levels rapidly decreased at 6 h and disappeared at 18 or 34 h upon treatment. However, when the plants were only deprived of carbon or nitrogen, a weak protein band could still be detected at 72 h for both ATG1a and ATg13a proteins (Suttangkakul, 2011, Plant Cell). These findings suggest that the degradation of ATG1a and ATG13a likely varied in response to different starvation conditions. In fact, our results (Figure 5E) showed that upon carbon or nitrogen treatment for 48 h, the levels of both ATG1a and ATG13a were similarly decreased, but not completely degraded, in comparison to that of the untreated control (0 h), which may be due to the separate carbon and nitrogen starvation treatments we used in our experiments. We have now included this information in Page 12 in the revised manuscript.

b) Several previous reports (Yoshimoto et al., 2004 Plant Cell 16: 2967; Suttangkakul, 2011, Plant Cell 23: 3761; Li et al., 2014 Plant Cell 26: 788) have suggested that on a normal SDS-PAGE gel, the two different type of bands detected by the anti-ATG8a antibodies correspond to different molecular sizes of ATG8s. In contrast, when the total proteins undergo a 6 M urea SDS gel (Yoshimoto et al., 2004 Plant Cell 16: 2967), or the separated membrane fraction is digested with PLD enzyme (Suttangkakul, 2011, Plant Cell 23: 3761; Li et al., 2014 Plant Cell 26: 788), the unmodified form ATG8s and the ATG8-PE conjugated form, the latter indicating the autophagic flux, could be detected by the anti-ATG8a antibodies. We therefore proposed that under carbon and nitrogen starvation conditions, the two different patterns of ATG8 bands

detected by the anti-ATG8a antibodies may be due to the activation of different ATG8 proteins. As suggested, we have now included this information on Page 12 in the revised manuscript.

Point 5. In Figure 6 A-D, using Y2H, CoIP and BiFC, they demonstrate that TRAF1A/B interacts with ATG6. For the CoIP and BiFC experiments, they should use another TRAF protein (one of the candidates they have tested, which did not relocate to autophagosomes), instead of empty vector. Xin lab speculated the presence of a TRAFosome in plants. Using another candidate, they could reveal if all TRAF proteins localize as a protein complex. Furthermore it is well known that empty vector controls are not the ideal controls in BiFC experiments (see Kudla& Bock, 2016, Plant Cell).

RESPONSE: We thank the reviewer for the very good suggestion. In the revised manuscript, we followed the reviewer's suggestion to include another TRAF protein, At4g01390, which did not translocate to the autophagosomes upon carbon starvation, as a negative control for the BiFC experiments. As shown in new Figure 6D, we observed that only TRAF1a, but not At4g01390, showed a physical interaction with ATG6 under light or dark conditions, which suggested that TRAF1 proteins identified in this study function in plant-specific TRAFosome formation and are essential for the regulation of autophagic dynamics in plants. We have now included this information on Page 13 in the revised manuscript.

Point 6. In Figure 6G, they look at TRAF1A and ATG6 levels under dark conditions. 24hrs after treatment, both TRAF1a and ATG6 disappear. Then they do MG132 and ConA treatment to block proteasome and autophagy, respectively. In MG132 treated samples, TRAF1a and ATG6 reappears, suggesting proteasome mediated degradation plays a role in their turnover. In ConA treated samples, we also see recovery of TRAF1A and ATG6 bands, although not as strong as MG132. So, the authors should revise this conclusion:

"Moreover, when dark-treated seedlings were treated with CA and MG132, the degradation of both TRAF1a-FLAG and ATG6-HA was strongly inhibited by MG132 (Figure 6G), suggesting that TRAF1a and ATG6 are highly regulated by the 26S proteasome in Arabidopsis cells.

RESPONSE: Thank you for your careful reviewing of our Figures. We agree with the reviewer's comments and have now revised the conclusion (Page 14) as follows:

"Moreover, when dark-treated seedlings were treated with CA and MG132, the degradation of both TRAF1a-FLAG and ATG6-HA was strongly inhibited by MG132 and CA (Figure 6G), suggesting that TRAF1a and ATG6 are highly regulated by the 26S proteasome and autophagy, the two dominant proteolytic pathways, in Arabidopsis cells."

Point 7. In Figure7B they have performed CoIP to test association of ATG6 with different SINAT E3 ligases. Based on this IP and Y2H assays, they conclude that ATG6 interacts with SINAT1, 2, 5S1, 5S2 and 6. However the CoIP experiment has several issues:

a) FLAG input blot is missing and by looking at unequal levels in the IP-FLAG blot, I guess SINATs are not expressed at similar levels. If this is the case, they should repeat these coIPs

b) although the authors say, SINAT3,4 don't interact with ATG6, I can see a band in the IP-HA blot for SINAT3 and 4?

RESPONSE: As suggested, we have now included the input blots for both tags in Figures 6C, 7B, and 8A in the revised manuscript. Particularly, we repeated the CoIP assay for Figure 7B, and tried our best to ensure equal protein loading in each lane. Our new data indicated that although the expression levels of SINAT1 and SINAT2 were slightly higher, the rest of the constructs, including SINAT3, SINAT4, SINAT5-S1 and SINAT6, were expressed at similar levels. Based on the CoIP results from this experiment and the other independent replicates, we concluded that SINAT5-S1, and SINAT6, but not SINAT3 and SINAT4, interact with ATG6.

Point 8. In figure 8E, the authors claim that SINAT6 prevents SINAT1-2 mediated degradation of ATG6 by measuring the intensity of the bands. This is one of the major findings of this paper. However the bands they measure are

saturated. They should repeat these WBs by loading less. Also they should do a dose dependent competition experiment with SINAT1 or SINAT2 and show that SINAT6 really prevents degradation of ATG6.

RESPONSE: Thank you very much for the constructive comments. In the revised manuscript, we followed the reviewer's suggestion to conduct a competition experiment to show the effects of SINAT6 on SINAT1 function. Our new data revealed that the various amounts of SINAT6 protein (0, 10, 20, and 30 □g plasmids) prevented the SINAT1-mediated degradation of ATG6 in a dose-dependent manner (new Figure 8E). We have now included this information in Page 17.

Point 9. Figure 8F shows protein levels of SINATs upon carbon starvation. SINAT1-2 levels decrease and SINAT6 levels increase upon starvation. In figure 6, they look at ATG6 levels upon starvation and show that ATG6 levels drop as early as 6 hours after starvation. The timings in these two figures seem to be contradictory. The authors should clarify this and discuss it in the discussion. Also at 24 hours post starvation, TRAFs seem to be degraded as well, so what is the physiological relevance of increased SINAT6 levels if there is no TRAF to recruit them?

RESPONSE: The reviewer is correct that SINAT1 and SINAT2 protein levels decreased and SINAT6 increased upon carbon starvation as early as 6 h after treatment (Figure 8F). However, the ATG6 levels increased at 6 h, followed by a rapid decline (Figure 6G). Given that the degradation of ATG6 protein was significantly inhibited by addition of CA and MG132 (Figure 6G), the starvation-induced degradation of ATG6 may indicate the activation of autophagy, suggesting that ATG6 may also function as both a regulator and a target for autophagy recycling in Arabidopsis cells. Meanwhile, the initial increase of ATG6 at 6 h may be due to the accumulation of SINAT6 protein and its prevention of SINAT1/2 function. Interestingly, a similar pattern, i.e. an initial increase followed by a rapid decline, was also observed in the starvation-induced changes of ATG13a protein (Suttangkakul, 2011, Plant Cell 23: 3761).

In contrast to their accumulation at 6 and 12 h upon starvation, the TRAF1 proteins were also degraded at 24 h post treatment (Figure 6G), suggesting that TRAF1 protein levels are highly regulated in different nutrient conditions and at different starvation stages. However, we observed that, different from the pattern of TRAF1s, the SINAT6 protein was continually activated from 6 to 24 h post starvation (Figure 8F). It is possible that SINAT6 may perform other functions rather than autophagy during nutrient starvation. We have included this information in the Discussion (Page 21) in the revised manuscript.

Point 10. TRAF1A and TRAF1B- are named Muse13 and Muse24. Since it is already published, the authors should use those names.

RESPONSE: Thank you very much for the comment. In the work of Huang et al. (2016 Cell Host & Microbe 19: 204), TRAF1a and TRAF1b proteins were isolated through forward screening for the snc1 enhancers and therefore designated as MUSE14 (Mutant, snc1-enhancing 14) and MUSE13, respectively. These names particularly referred to the function of these two proteins in the turnover of plant autoimmunity receptors. However, in this study, we used reverse genetics to screen the TRAF-family proteins with potential roles in autophagy by their starvation-inducible autophagosome translocation in Arabidopsis cells through confocal microscopy (Figure 1). Given that the Arabidopsis genome has more than 70 TRAF-family proteins which may have diverse physiological functions rather than autoimmunity and autophagy in plant cells, we therefore think it should be more reasonable to rename these two proteins TRAF1a and TRAF1b, which is more convenient for the researchers to designate and identify the other 70 TRAF-containing proteins. We have referred to the original names in the text (Pages 2, 5, and 20) to avoid confusion in the literature.

Reviewer #2:

The manuscript by Qi and co-authors shows that two TRAF proteins, TRAF1a and b, together with the RING E3 ligases SINAT1, SINAT2, and SINAT6 affect the ubiquitination dynamics of the autophagy protein ATG6. By combining mutant analysis and biochemistry, the authors show that TRAF1a and b interact and colocalize with ATG6 during autophagy. During conditions that trigger autophagy (starvation), SINAT1 and 2 ubiquitinate ATG6 in the presence of the TRAF proteins, leading to its degradation, whereas SINT6 has the opposite effect.

The paper presents a large amount of data and contributes important insights on the regulation of ATG6 in plants. However, there are several points that the authors should address to strength their work:

Point 1. The major problem in that the connection between phenotypic alterations of the *traf* mutants and autophagy is rather weak. The only experiment to determine that TRAF1a and b are required for autophagosome formation is the quantification of autophagosomes under conditions that favor autophagy. Considering the highly pleiotropic defects of the double *traf* mutant due to constitutive pathogen response, quantifying puncta in cells with obvious defects in expansion/growth is not reliable. The authors have already introduced eGFP-ATG8 in the *traf* mutants. A western blot showing the ratio between eGFP-ATG8 to free GFP cleaved from the full protein upon delivery to the vacuole would provide a much more quantitative assessment of autophagy fluxes. This should also be done in the *sinat* mutants.

RESPONSE: Thank you very much for your constructive suggestions. As suggested, we provided new evidence (Supplemental Figures 9 and 13A) to show that, consistent with the confocal data, the starvation-inducible releases of free eGFP from eGFP-ATG8e were significantly reduced in the *traf1a/b* and *sinat6* mutants, but significantly enhanced in the SINAT1/2-KO mutants, compared to that of wild type background. We have now included these results on Pages 12 and 18 in the revised manuscript.

Point 2. The manuscript is hard to read. The first part of the Results shows in detail alterations of the *traf* mutants that are clearly linked to a constitutive pathogen response. A recent paper by Huang et al (2016) already reported that TRAF1a and b are needed for the turnover of immunoreceptors. It is unclear what the authors try to convey with this data. Are the responses they see (spontaneous cell death, upregulation of defense genes, etc.) related to autophagy or not?

RESPONSE: Thank you for the comments. We have now tried our best to improve the manuscript, as suggested. Previous reports demonstrate that all of the autophagy-deficient *atg* mutants showed constitutive pathogen responses, including enhanced SA and JA levels, accumulation of reactive oxygen species (ROS), spontaneous cell death, upregulation of defense genes, as well as altered sensitivities to pathogen infection (Yoshimoto et al., 2009 Plant Cell 21: 2914; Lenz et al., 2011 Plant J. 66: 818; Lai et al., 2011 Plant J. 66: 953; Wang et al., 2011 Plant J. 68: 74). The reviewer is correct that a recent paper by Huang et al. (2016 Cell Host & Microbe 19: 204) already showed that TRAF1s (MUSE13 and MUSE14) are needed for the turnover of immunoreceptors and their knockout double mutants are more resistant to pathogens such as *Hyaloperonospora arabidopsidis* Noco2 and *Pseudomonas syringae* pv. Tomato (P. st.) DC3000 with AvrRpt2. Here, we performed more detailed investigations of TRAF1-mediated defense responses in the *traf1a/b* mutants by determining the increased SA and JA contents, the accumulation of ROS and constitutive cell death, activated expression of defense responsive genes, and increased and decreased susceptibilities to pathogens *Botrytis cinerea* and P. st. DC3000, respectively; these observations are comparable to those of *atg* mutants.

Point 3. Coomassie gels should not be used as loading controls for western blots. They do not have enough sensitivity for the mild changes in protein abundance detected with antibodies.

RESPONSE: We agree with the reviewer's comment that Coomassie-stained gels may not be the best controls for the mild changes of protein abundance in western blots. However, for all of the western blot analyses in this study, we have ensured the absolute equal loading for all samples and significant changes could be observed in the mutants or treated time points in comparison with the control groups (Figures 5E, 6E-G, 7F-G, and 8B-F). To further ensure the successful transfer of proteins from the gels to the membranes, we also used the Ponceau S to stain the membranes before western blot analyses. Throughout the revised manuscript, we therefore replaced the Coomassie-stained gels with the Ponceau-staining membranes, which should directly reflect the exact amounts of proteins that reacted with the antibodies and were previously used as loading controls for western blot analyses (Hackenberg et al., 2013 Plant Cell 25: 4616; Munch et al., 2015 Plant Cell 27: 463; Nhuiyan et al., 2016 Plant Cell 28: 3020).

Point 4. Figure 6F: This is transient transformation assay with ATG6-HA in protoplasts, in the presence of CHX. At time 0, there is already more ATG6-HA in WT protoplast than in *traf1a;b* or *traf1a;b* TRAF1a-FLAG. The authors

should start from the same amount of ATG6-HA for this experiment to be meaningful. The loading control is GFP. What is the GFP coming from? Why is it not degraded during the CHX treatment?

RESPONSE: Thank you for your careful review of our Figures. The reviewer is correct that in the untreated samples (0 h), the ATG6-HA expression levels are always higher in the *traf1a/b* mutant and TRAF1a-FLAG than in wild type (new Figure 6F). These observations were reproducible in all of three independent experiments. Given that: 1) the total proteins loaded were equal in all of samples, 2) the degradation of ATG6-HA was evident after 10, 20, and 30 min of CHX treatment in wild-type cells, but not in *traf1a/b-1* cells, we therefore believe that the lower expression level of ATG6-HA in WT cells at time 0 is due to the nature of its stabilities. We have now included this information in Page 13 in the revised manuscript.

In the original Figure 6F, the GFP plasmid was co-expressed as an internal expression control, which is stable during the CHX treatment. In the revised manuscript, we followed the reviewers' suggestions to use the Ponceau-stained membranes as loading controls for all of the blots.

Point 5. Fig 7F: Why is there an unspecific band only in the vector only control? How can the authors explain that this band does not show in any of the other lanes?

RESPONSE: Yes, there is an unspecific band in the cells co-expressing ATG6-HA and vector control, but not in the cells co-expressing ATG6-HA and SINATs-FLAG (Figure 7F). It may represent an unidentified modification of ATG6-HA in the absence of SINATs. Interestingly, a similar band was also observed in SINA3-targeted ubiquitination of transcription factor NAC1 involved in plant defense response (Miao et al., 2016 New Phytologist 211: 138). We therefore added the following sentence on Page 15 to clarify this:

"We note that in the absence of SINATs, the immunoblot showed an unspecific band (indicated by the asterisk in Figure 7F), which may represent an unidentified modification of ATG6-HA. The nature of this unspecific band remains to be determined."

Point 6. The authors should discuss more in detail about how ubiquitination modulate the function in Beclin-1/ATG6 in other organisms

RESPONSE: As suggested, we have now added more information on the function of Beclin-1/ATG6 ubiquitination in autophagy in mammals in Pages 4, 5, and 20.

Point 7. TRAF1a and b are already annotated in TAIR as MUSE13 and 14 according to Huang et al (2016). Is there a particular reason for not using these names?

RESPONSE: Thank you very much for the comment. In the work of Huang et al. (2016 Cell Host & Microbe 19: 204), TRAF1a and TRAF1b proteins were isolated through forward screening for the snc1 enhancers and therefore designated as MUSE14 (Mutant, snc1-enhancing 14) and MUSE13, respectively. These names particularly referred to the function of these two proteins in the turnover of plant autoimmunity receptors. However, in this study, we used reverse genetics to screen the TRAF-family proteins with potential roles in autophagy by their starvation-inducible autophagosome translocation in Arabidopsis cells through confocal microscopy (Figure 1). Given that the Arabidopsis genome has more than 70 TRAF-family proteins which may have diverse physiological functions rather than autoimmunity and autophagy in plant cells, we therefore think it should be more reasonable to rename these two proteins as TRAF1a and TRAF1b, which is more convenient for the researchers to designate and identify the remaining 70 TRAF-containing proteins.

TPC2017-00056-RAR1 2nd Editorial decision – *acceptance pending* March 16, 2017

We are pleased to inform you that your paper entitled "The TRAF-Family Proteins TRAF1a and TRAF1b Regulate Autophagy Dynamics by Modulating ATG6 Stability in Arabidopsis 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	**March 27, 2017**