nature portfolio

Peer Review File

Autophagy modulates Arabidopsis male gametophyte fertility and controls actin organization

Corresponding Author: Professor Hao WANG

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The article entitled “Autophagy regulates Arabidopsis male gametophyte development and fertility by controlling actin organization” describing the role of autophagy during plant sexual reproduction, and particularly pay attention to sperm cell biogenesis. By analyzing reproduction in atg5 and atg7 mutants, the authors demonstrated that autophagy does in fact play an essential role in regulating male gametophyte development and fertility in Arabidopsis, and found that reduced autophagy activity in germinated pollen accompanied by partial aberrations in sperm cell biogenesis and pollen tube growth, leading to compromised seed formation. To characterize the cellular defects in pollen of atg5 and atg7 mutants, the authors found that sperm cell biogenesis defects is associated with alteration in the actin cytoskeleton in pollen. The authors further demonstrated that the alterations in the actin cytoskeleton presumably due to the failure in the degradation of actin depolymerizing factors ADF7 and Profilin2. The authors also demonstrated that Neighbor of BRCA1 (NBR1) acts as a key receptor to interact with ADF7 and Profilin2 for degradation in a manner independent of ubiquitination. The authors thus proposed that autopahgy regulates sperm cell biogenesis and male fertility by controlling actin organization in Arabidopsis. Overall, it is an interesting study, but more experiments need to be done to provide mechanistic insights into how autophagy regulates actin organization in pollen.
1. The authors need to demonstrate that the failure in the degradation of ADF7 and Profilin2 indeed accounts for the alteration in the actin cytoskeleton and sperm cell biogenesis in atg5 and atg7 mutant pollen. Right now, the authors only have data to show that overexpression of ADF7 to some extent phenocopies the sperm cell biogenesis defects in atg5 and atg7 mutant pollen. The authors might want to use reverse genetic approach to demonstrate whether depletion of ADF7 or Profilin2 can suppress the phenotype associated with atg5 and atg7 mutants.
2. To make a tight link between autophagy and the actin cytoskeleton, this reviewer will suggest the authors to analyze the phenotype associated with the loss of function of NBR1.
3. Furthermore, if the authors can map the binding site of NBR1 in ADF7 and Profilin2, put back the mutant ADF7 and Profilin2 that fail to interact with NBR1 by mutating the binding site(s) into their own mutant to see what happen to the actin cytoskeleton in pollen will be extremely interesting.
4. In addition, the authors should clarify how they determine that it is ADF7 and Profilin2, not other members from the same family, which are targeted by autophagy?

Reviewer #2

(Remarks to the Author)
The article addresses an interesting scientific problem – the factors regulating the sperm cells biogenesis and the pollen tube growth. However, the authors conclusion that autophagy regulates gametophyte development and fertility by controlling actin organization due to selective degradation of ADF7 and PRF2 is not sufficiently documented. The presented concept is interesting but data are too preliminary and additional evidence is needed. The project rationale consists of the following observations: (1) reduced autophagy flux in germinating pollen of atg5 and atg7 mutants than of WT, and colocalization of ATG5 with ATG7, ATG6, ATG8e, ATG9 and SH3P2; (2) partially defective seed formation and pollen tube germination (in vitro and in vivo) in atg5 and atg7 mutants; (3) delayed pollen tube growth and pollen germination rate and initial stages, undivided generative nucleus and twisted pollen tubes in a about 20% of atg5 and atg7 mutants; (4) higher depolymerization of actin filaments in pollen grains of atg5 and atg7 mutants than in WT.
Finally, the authors identify ADF7 (actin depolymerizing factor 7) and PRF2 (profilin 2) as partners of NBR1, a selective

autophagy cargo receptor. They propose that the failure of autophagic degradation of these proteins leads to actin depolymerization in the atg5 and atg7 mutants. The observations 1-4 are illustrated by the good quality images, the presented data are convincing. However, the explanation of the mechanism responsible for the observed affects is not sufficiently documented and raises even more questions. (a) It is necessary to provide more genetic evidence, for example by checking the phenotype of the nbr1 mutant; (b) both ADF7 and PRF2 are members of larger families. Do other members of these families interact with NBR1? (c) Actin reorganization is a dynamic process with numerous signals and factors involved in its regulation, such as calcium influx and gradients, ARP2/3 complex and other actin binding proteins. Is the homeostasis of the other factors changed in atg5 and atg7? (d) Demonstration of interaction of NBR1 with ADF7 and PRF2 is not sufficient proof for the selective targeting of these proteins to autophagy via NBR1. You should verify if autophagy inhibitors affect the degradation of ATG7 and PRF2. (e) Materials and methods section lacks the precision and necessary details in many places.
Some specific comments:
1) It should be RT-qPCR not qRT-PCR (Results/ Methods) because it is Reverse Transcription- quantitative PCR
2) In Fig.1a mark the positions of the primers used in 1b-d. The transcript level detected in T-DNA mutants could be different depending on the position of the primers.
3) l.132-133 – it is not clear from Suppl. Fig.1 that ATG7 is enriched in sperm cells
4) provide more information about the antibodies in Methods; anti-ATG8 are only mentioned (no source and specific reference; e.g. l. 479-480), anti-profilin and anti-ADF7 are even not mentioned in the Methods.
5) explain LatB in the legend to Fig.4

Reviewer #3

(Remarks to the Author)

In their manuscript, Yan et al. have revisited the potential repercussions of deficient (macro)autophagy on spermatogenesis and the directional growth of pollen. Their findings indicate that the absence of autophagic degradation of actin filament depolymerization factors ADF7 and Profilin2 leads to an elevated incidence of defective sperm cell biogenesis. Moreover, this deficiency has an impact on the directional growth of pollen tubes during fertilization, culminating in the formation of relatively moderate abortive seeds within the Arabidopsis silique.

Major remarks and comments:

General comment: The manuscript exhibits moments of confusion and scientific perplexity, especially concerning the design and scientific underpinnings of specific experiments and the interpretation of observed results. The title of some of the figures are scientifically confusing (for example Fig.3 and Fig. 5). Clarity and coherence need to be enhanced for a more effective and impactful communication of the research findings. It would be helpful considering revisiting the experimental descriptions and data interpretation to ensure a more straightforward and scientifically sound presentation.

Point remarks on the main figures:

1. Fig. 1:

Panel b, c, d: The method used to assess the relative expression of ATG5 and ATG7 in different genetic backgrounds is unclear, especially as the expression in the wild-type appears almost equal to 1 in all cases. Could you clarify if this is relative to actin expression?

Panel e: Adding a molecular mass marker would enhance the interpretability of the results.

Panel f: It would be beneficial if the authors could provide a discussion on the nature of the various punctuate structures labeled by each fluorescent fusion protein. Understanding the characteristics of these structures would contribute to the overall comprehension of the findings.

Additionally, it's not clear why the authors did not investigate the complementation of the atg5 and atg7 mutant phenotype in the Arabidopsis male gametophyte with the corresponding fluorescent chimera. Addressing this aspect would strengthen the study by providing a more comprehensive understanding of the observed phenotypes.

2. Fig. 2:

Panel c-f: There may be a potential correlation between the observed reduction in pollen tube growth and the obvious delay in germination of mutant pollen grains. Notably, all genotypes exhibit similar pollen tube lengths after 10 hours. It would be interesting for the authors to discuss this observation further, exploring the potential interplay between pollen grain germination and subsequent tube growth. Providing insights into the dynamics of these processes could enhance the overall understanding of the experimental outcomes.It is not clear why the authors did not also test pollen grains from the double mutant atg5/atg7.

3. Fig. 4:

The data suggests that autophagy deficiency, linked to the lack of function of ATG5 or ATG7, influences actin filament

depolymerization and potentially impacts directional/polarized growth. It would be valuable for the authors to discuss whether this observed phenotype is specific to pollen tube growth or if other cell types, such as root hairs, exhibit similar effects. Expanding the discussion to include insights into the broader implications of autophagy deficiency on different cell types would provide a more comprehensive perspective on the study's findings.

4. Fig. 5:

Panel a: The western blot bands for both anti-ADF and anti-Profilin appear saturated, posing challenges for their relative quantification. Consider addressing this issue or providing alternative quantification methods to enhance the accuracy of the data.

Panel d: There appears to be a faint band around ~100 kDa in the control lane (GFP) in the ADF panel. It would be beneficial for the authors to discuss this observation and explore its potential implications. Additionally, the relatively high level of free GFP compared to full-length GFP-NBR1 raises questions about the stability of GFP-NBR1 and the efficiency of coIP. A discussion on these observations would contribute to a better understanding of the stability of the tagged protein and the efficiency of coimmunoprecipitation.

Can NBR1 binds simultaneously ADF7 and Profilin2?

5. Fig.6: The proposed model requires clarification. Specifically, it is inaccurate to suggest that in the atg5 or atg7 mutant, the phagophore fails to generate a closed autophagosome. Please revisit and refine the model to accurately represent the observed findings and avoid potential misconceptions regarding the role of autophagy in phagophore development in the mutants.

Minor point : Refining the entire manuscript, including figure legends, for both scientific and linguistic clarity would be helpful.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This reviewer is not convinced that autophagy regulates the actin cytoskeleton in pollen via targeting and degrading ADF7 and Profilin2, as the authors stated that NBR1 depletion does not show obvious actin phenotype in pollen. In addition, this authors did not agree with the authors that characterization of the interaction of BBR1 with ADF7 and Profilin2 is beyond the scope of this study. This reviewer actually believes that it is an essential data to make this story publishable in the top-notched journal like Nature Communications. Furthermore, this reviewer also has a minor concern about the description in Supplemental Figure 5, in which the authors simply grouped profilin as actin-depolymerizing factor might be inappropriate.

Reviewer #2

(Remarks to the Author)
The article addresses an interesting scientific problem – the factors regulating sperm cell biogenesis and pollen tube growth.
The authors addressed my comments and answered the questions.
Additional experiments were performed. The methodology is now explained sufficiently. The revision is satisfactory.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily revised their manuscript, addressing my comments and concerns effectively.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My concern has been fully addressed in this revised manuscript. I therefore have no further comments on this manuscript and I am now happy to recommend it for publication in Nature Communications.

Response to reviewers

Reviewer #1 (Remarks to the Author):

The article entitled “Autophagy regulates Arabidopsis male gametophyte development and fertility by controlling actin organization” describing the role of autophagy during plant sexual reproduction, and particularly pay attention to sperm cell biogenesis. By analyzing reproduction in atg5 and atg7 mutants, the authors demonstrated that autophagy does in fact play an essential role in regulating male gametophyte development and fertility in Arabidopsis, and found that reduced autophagy activity in germinated pollen accompanied by partial aberrations in sperm cell biogenesis and pollen tube growth, leading to compromised seed formation. To characterize the cellular defects in pollen of atg5 and atg7 mutants, the authors found that sperm cell biogenesis defects is associated with alteration in the actin cytoskeleton in pollen. The authors further demonstrated that the alterations in the actin cytoskeleton presumably due to the failure in the degradation of actin depolymerizing factors ADF7 and Profilin2. The authors also demonstrated that Neighbor of BRCA1 (NBR1) acts as a key receptor to interact with ADF7 and Profilin2 for degradation in a manner independent of ubiquitination. The authors thus proposed that autophagy regulates sperm cell biogenesis and male fertility by controlling actin organization in Arabidopsis. Overall, it is an interesting study, but more experiments need to be done to provide mechanistic insights into how autophagy regulates actin organization in pollen.

Response: Thank you very much for your constructive suggestions and comments on our manuscript.

1. The authors need to demonstrate that the failure in the degradation of ADF7 and Profilin2 indeed accounts for the alteration in the actin cytoskeleton and sperm cell biogenesis in *atg5* and *atg7* mutant pollen. Right now, the authors only have data to show that overexpression of ADF7 to some extent phenocopies the sperm cell biogenesis defects in atg5 and atg7 mutant pollen. The authors might want to use reverse genetic approach to demonstrate whether depletion of ADF7 or Profilin2 can suppress the phenotype associated with atg5 and atg7 mutants.

Response: We have down-regulated the expression of *ADFs* and *Profilins* by generation of *ADF*-RNAi and *Profilin*-RNAi transgenic Arabidopsis lines in both *atg5-1* and *atg7-2* backgrounds. Our findings indicate that the seed setting defects observed in the *atg5-1* and *atg7-2* mutants were partially restored by approximately 8% and 5% in the *ADF*-RNAi and *Profilin*-RNAi transgenic plants respectively. Please see the new Supplementary Fig. 11. We also included related text in the manuscript, please see line 338-346, 505-508 and 530-532.

2. To make a tight link between autophagy and the actin cytoskeleton, this reviewer will suggest the authors to analyze the phenotype associated with the loss of function of NBR1.

Response: We have carefully analyzed phenotypes of the *nbr1-2c* mutant by observing the sperm cell formation, actin cytoskeleton organization in pollen grains and pollen tubes and calculating its seed setting rate. The *nbr1-2c* was generated and studied as the previous study described[1]. We found no significant phenotypic differences between *nbr1-2c* and the WT. Please see the new Supplementary Fig.12. We have also included the related text in the manuscript, please see line 348-357.

3. Furthermore, if the authors can map the binding site of NBR1 in ADF7 and Profilin2, put back the mutant ADF7 and Profilin2 that fail to interact with NBR1 by mutating the binding site(s) into their own mutant to see what happen to the actin cytoskeleton in pollen will be extremely interesting.

Response: We agreed with your suggestion and it is essential to understand the molecular details of how NBR1 recognizes cargo proteins. Actually, we have mapped and studied the binding sites of NBR1 with cargo proteins in detail using various approaches. However, we have finalized and included our results in a new manuscript which has been submitted to a peer-reviewed journal. The manuscript is currently under revision. Consequently, we hope you will agree with us that identification of the binding sites of NBR1 with ADF7 and Profilin2 is beyond the scope of this study despite of its importance.

4. In addition, the authors should clarify how they determine that it is ADF7 and Profilin2, not other members from the same family, which are targeted by autophagy?

Response: We have performed a systematic screening and analysis of the interactions between NBR1 with ADF and Profilin family proteins respectively. Please see the new Supplementary Fig. 5 and 7. We firstly selected the ADFs and Profilins that function in actin depolymerization as show in Supplementary Fig. 5. For ADF family proteins, we employed luciferase complementation assay to screen the interactions between NBR1 and ADFs and identified that ADF7 showed the strongest interaction with NBR1 despite weak protein interactions were also detected between NBR1 with other ADFs. Please see the new Supplementary Fig. 7a and b. Furthermore, we checked various ADF gene expression profiles from TAIR (https://www.arabidopsis.org/) and found that only *ADF7* and *ADF10* are highly expressed in pollens. Please see the new Supplementary Fig. 7c. Based on these results, ADF7 is determined and used in our study. For Profilin family proteins, only Profilin2 interacts with NBR1 in the luciferase complementation assay. Please see new Supplementary Fig. 7d. We have also included related detailed information in our manuscript, please see line 286-299.

References

1. Ji, C. *et al.* AtNBR1 Is a Selective Autophagic Receptor for AtExo70E2 in Arabidopsis. *Plant Physiol* **184**, 777-791 (2020).

Reviewer #2 (Remarks to the Author):

The article addresses an interesting scientific problem – the factors regulating the sperm cells biogenesis and the pollen tube growth. However, the authors conclusion that autophagy regulates gametophyte development and fertility by controlling actin organization due to selective degradation of ADF7 and PRF2 is not sufficiently documented. The presented concept is interesting but data are too preliminary and additional evidence is needed. The project rationale consists of the following observations: (1) reduced autophagy flux in germinating pollen of atg5 and atg7 mutants than of WT, and colocalization of ATG5 with ATG7, ATG6, ATG8e, ATG9 and SH3P2; (2) partially defective seed formation and pollen tube germination (in vitro and in vivo) in atg5 and atg7 mutants; (3) delayed pollen tube growth and pollen germination rate and initial stages, undivided generative nucleus and twisted pollen tubes in a about 20% of atg5 and atg7 mutants; (4) higher depolymerization of actin filaments in pollen grains of atg5 and atg7 mutants than in WT. Finally, the authors identify ADF7 (actin depolymerizing factor 7) and PRF2 (profilin 2) as partners of NBR1, a selective autophagy cargo receptor. They propose that the failure of autophagic degradation of these proteins leads to actin depolymerization in the atg5 and atg7 mutants. The observations 1-4 are illustrated by the good quality images, the presented data are convincing.
Response: Thank you very much for your constructive suggestions and comments on our manuscript.

However, the explanation of the mechanism responsible for the observed affects is not sufficiently documented and raises even more questions.
(a) It is necessary to provide more genetic evidence, for example by checking the phenotype of the nbr1 mutant;
Response: We have performed additional genetic studies to further improve our study including: i) we have observed and compared the pollen tube actin organization and seed setting rate between the *nbr1-2c* mutant and the WT. However, no significant differences were observed. Please see the new Supplementary Fig.12. We also discussed these results in the manuscript, please see line 348-357; ii) we have generated *atg5-1* and *atg7-2* complementary lines and found that the male reproductive defects of *atg5-1* and *atg7-2* can be successfully recovered. Please see revised Fig. 2a and b and line 168-171; and iii) to further examine the interplays between actin and autophagy, we have generated *ADF*-RNAi and *Profilin*-RNAi transgenic Arabidopsis in *atg5-1* and *atg7-2* backgrounds to down-regulate the expression of *ADFs* and *Profilin2*. Our findings indicate that the seed setting defects observed in the *atg5-1* and *atg7-2* mutants were partially restored by approximately 8% and 5% in the *ADF*-RNAi and *Profilin*-RNAi transgenic plants respectively. Please see the new Supplementary Fig. 11 and related new manuscript text in line 338-346, 505-508 and 530-532.

(b) both ADF7 and PRF2 are members of larger families. Do other members of these families interact with NBR1?

Response: We have performed a systematic screening and analysis of the interactions between NBR1 with ADF and Profilin family proteins respectively. Please see the new Supplementary Fig. 5 and 7. We firstly selected the ADFs and Profilins that function in actin depolymerization as show in Supplementary Fig. 5. For ADF family proteins, we employed luciferase complementation assay to screen the interactions between NBR1 and ADFs and identified that ADF7 showed the strongest interaction with NBR1 despite weak protein interactions were also detected between NBR1 with other ADFs. Please see the new Supplementary Fig. 7a and b. Furthermore, we checked various ADF gene expression profiles from TAIR (https://www.arabidopsis.org/) and found that only *ADF7* and *ADF10* are highly expressed in pollens. Please see the new Supplementary Fig. 7c. Based on these results, ADF7 is determined and used in our study. For Profilin family proteins, only Profilin2 interacts with NBR1 in the luciferase complementation assay. Please see new Supplementary Fig. 7d. We have also included related detailed information in our manuscript, please see line 286-299.

(c) Actin reorganization is a dynamic process with numerous signals and factors involved in its regulation, such as calcium influx and gradients, ARP2/3 complex and other actin binding proteins. Is the homeostasis of the other factors changed in atg5 and atg7?

Response: We have performed additional experiments to detect the calcium gradient in growing pollen tubes and protein interactions between NBR1 and ARP2/3. Please see the new Supplementary Fig. 8. Our results showed that the tip-focused calcium gradient was disrupted in *atg5-1* and *atg7-2* mutants. We have also included the related text in the manuscript, please see line 305-309. In addition, we have detected protein interaction between ARP2/3 and NBR1 by luciferase complementation assay. Please see the new Supplementary Fig. 9. However, we found that the expression level of *ARP2* and *3* in Arabidopsis pollens is extremely low from TAIR (https://www.arabidopsis.org/). Thus, ARP2/3 may not play a central role in regulating the pollen tube actin dynamics in *atg5-1* and *atg7-2* despite its interaction with NBR1. We have also included the related text in the manuscript, please see line 493-500.

(d) Demonstration of interaction of NBR1 with ADF7 and PRF2 is not sufficient proof for the selective targeting of these proteins to autophagy via NBR1. You should verify if autophagy inhibitors affect the degradation of ADF7 and PRF2.

Response: We have performed additional pharmaceutical treatments by applying autophagy inhibitors 3-Methyladenine and wortmannin on the WT mature pollens. The amount of ADF and Profilin proteins greatly increased

after inhibition of autophagy. Please see the new Supplementary Fig. 6. In addition, we have included the related information in the manuscript, please see line 278-282.

Materials and methods section lacks the precision and necessary details in many places.
Response: We have carefully revised the Materials and Methods section by adding more detailed information, please see line 524-541, 573-575, 577, 580, 583, 585, 632, 639, 643-644, 656, 661 and 672-674.

Some specific comments:
1) It should be RT-qPCR not qRT-PCR (Results/ Methods) because it is Reverse Transcription- quantitative PCR
Response: We have made corrections accordingly. Please see line 123, 577, 580 and 861.

2) In Fig.1a mark the positions of the primers used in 1b-d. The transcript level detected in T-DNA mutants could be different depending on the position of the primers.
Response: We have marked the positions of the primers in Fig. 1a. Please see the revised Fig. 1a and line 860-861.

3) l.132-133 – it is not clear from Suppl. Fig.1 that ATG7 is enriched in sperm cells
Response: We have made corrections to clarify the statement in the text. Please see line 130.

4) provide more information about the antibodies in Methods; anti-ATG8 are only mentioned (no source and specific reference; e.g. l. 479-480), anti-profilin and anti-ADF7 are even not mentioned in the Methods.
Response: We have added more detailed information about anti-ATG8, anti-Profilin, anti-ADF, anti-GFP and anti-NBR1 antibodies. Please see line 573-575 and 672-673.

5) explain LatB in the legend to Fig.4
Response: Done. Please see line 922-923.

Reviewer #3 (Remarks to the Author):

In their manuscript, Yan et al. have revisited the potential repercussions of deficient (macro)autophagy on spermatogenesis and the directional growth of pollen. Their findings indicate that the absence of autophagic degradation of actin filament depolymerization factors ADF7 and Profilin2 leads to an elevated incidence of defective sperm cell biogenesis. Moreover, this deficiency has an impact on the directional growth of pollen tubes during fertilization, culminating in the formation of relatively moderate abortive seeds within the Arabidopsis silique.

Response: Thank you very much for your constructive suggestions and comments on our manuscript.

Major remarks and comments:

General comment: The manuscript exhibits moments of confusion and scientific perplexity, especially concerning the design and scientific underpinnings of specific experiments and the interpretation of observed results. The title of some of the figures are scientifically confusing (for example Fig.3 and Fig. 5). Clarity and coherence need to be enhanced for a more effective and impactful communication of the research findings. It would be helpful considering revisiting the experimental descriptions and data interpretation to ensure a more straightforward and scientifically sound presentation.

Response: We have carefully revised the manuscript including: i) the titles of the figures as you suggested. Please see line 901-903 and 931-932.; ii) carefully revised the Results and Discussion to improve clarification and rationale of our study. Please see line 123, 168-171, 203, 250-260, 278-282, 286-299, 305-309, 338-346, 348-357, 397-407, 440-448, 493-500 and 505-508; and iii) revised the figures and conducted additional experiments to strengthen the scientific robustness of our study. Please see the revised Fig.1, 2, 5 and 6, newly generated Supplementary Fig. 3, 4, 6, 7, 8, 9, 11 and 12.

Point remarks on the main figures:

1. Fig. 1:

Panel b, c, d: The method used to assess the relative expression of ATG5 and ATG7 in different genetic backgrounds is unclear, especially as the expression in the wild-type appears almost equal to 1 in all cases. Could you clarify if this is relative to actin expression?

Response: We previously normalized the expression levels of *ATG5* and *ATG7* in the WT background as 1 to compare with various mutants. To better clarify the results, we now have revised the figure by using *Actin2* as the internal control. Please see the revised Fig. 1b and c.

Panel e: Adding a molecular mass marker would enhance the interpretability of the results.
Response：We have added a molecular marker in Fig. 1d as you suggested. Please see the revised Figure 1d.

Panel f: It would be beneficial if the authors could provide a discussion on the nature of the various punctuate structures labeled by each fluorescent fusion protein. Understanding the characteristics of these structures would contribute to the overall comprehension of the findings.
Response: We have included a brief discussion on the nature of the various punctuate structures in the Discussion as suggested. Please see line 397-407.

Additionally, it's not clear why the authors did not investigate the complementation of the atg5 and atg7 mutant phenotype in the Arabidopsis male gametophyte with the corresponding fluorescent chimera. Addressing this aspect would strengthen the study by providing a more comprehensive understanding of the observed phenotypes.
Response: it is time-consuming to generate genetic Arabidopsis co-expression of various autophagy marker proteins together with fluorescent tagged ATG5 and ATG7. Instead, biolistic transient co-expression of multiple chimeric fluorescent fusion proteins has been well documented as a robust and reliable method to investigate protein subcellular localizations and dynamics in pollens and growing pollen tubes[1, 2]. Thus, we hope you will agree with us that it is convincing enough of using biolistic transient expression to study protein subcellular localization in pollen tubes. In addition, we actually have generated genetic complementary lines (*atg5-1 COM#* and *atg7-2 COM#*) by native promoter induced expression of chimeric fusion of fluorescent protein with ATG5 and ATG7 in the *atg5* and *atg7* mutants respectively. We studied the phenotypes of male gametophyte using the complementary lines. The defects of the mutants can be recovered. These new results have been included in Fig. 2a and b. Please see the revised Fig. 2a and b.

2. Fig. 2:
Panel c-f: There may be a potential correlation between the observed reduction in pollen tube growth and the obvious delay in germination of mutant pollen grains. Notably, all genotypes exhibit similar pollen tube lengths after 10 hours. It would be interesting for the authors to discuss this observation further, exploring the potential interplay between pollen grain germination and subsequent tube growth. Providing insights into the dynamics of these processes could enhance the overall understanding of the experimental outcomes. It is not clear why the authors did not also test pollen grains from the double mutant atg5/atg7.
Response: We have a included a discussion on the potential interplays between the defects of pollen tube growth and pollen germination in the Discussion as you suggested. Please see line 439-447. On the other hand, we

have included the data of *in vivo* and *in vitro* pollen germination of the *atg5-1*/*atg7-2* double mutant. Please see the revised Fig. 2c-g and Supplementary Fig. 3.

3. Fig. 4:
The data suggests that autophagy deficiency, linked to the lack of function of ATG5 or ATG7, influences actin filament depolymerization and potentially impacts directional/polarized growth. It would be valuable for the authors to discuss whether this observed phenotype is specific to pollen tube growth or if other cell types, such as root hairs, exhibit similar effects. Expanding the discussion to include insights into the broader implications of autophagy deficiency on different cell types would provide a more comprehensive perspective on the study's findings.
Response: We have compared the phenotypes of root hair, root and leaf pavement cells between the WT and *atg5* or *atg7*. We only found that the root hairs are much shorter and actin organization is depolymerized in the mutants. Please see the new Supplementary Fig. 4. We also included a brief discussion on the observed phenotypes. Please see line 250-259.

Panel a: The western blot bands for both anti-ADF and anti-Profilin appear saturated, posing challenges for their relative quantification. Consider addressing this issue or providing alternative quantification methods to enhance the accuracy of the data.
Response: We have re-conducted the experiment to avoid over saturation of the bands for quantification. Please see the revised Fig. 5a.

Panel d: There appears to be a faint band around ~100 kDa in the control lane (GFP) in the ADF panel. It would be beneficial for the authors to discuss this observation and explore its potential implications. Additionally, the relatively high level of free GFP compared to full-length GFP-NBR1 raises questions about the stability of GFP-NBR1 and the efficiency of coIP. A discussion on these observations would contribute to a better understanding of the stability of the tagged protein and the efficiency of coimmunoprecipitation.
Response: It is likely that the faint band around ~100 kDa in the control lane is a non-specific band by using anti-GFP antibody. Unfortunately, the size of this non-specific band is the same size with the target band of GFP-NBR1 after Co-IP. To avoid misunderstanding and better clarify the Co-IP result, we re-performed it by using GFP-ADF7 and GFP-Profilin2 over-expressing Arabidopsis with newly obtained anti-NBR1 antibody. Please see the revised Fig. 5d.

Can NBR1 binds simultaneously ADF7 and Profilin2?
Response：We have employed AlphaFold to predict the simultaneous binding ability of NBR1 with multiple types of cargo proteins. The result showed that NBR1 is not able to bind to two or more different types of cargoes at the same

time. Furthermore, we have characterized the functional binding sits of NBR1 with cargo proteins by various approaches, and finalized these results in a new manuscript which has been submitted to a peer-reviewed journal. The manuscript is currently under revision. Consequently, we hope you will agree with us that identification of the binding sites of NBR1 and its binding ability with both ADF7 and Profilin2 is beyond the scope of this study despite of its importance.

5. Fig.6: The proposed model requires clarification. Specifically, it is inaccurate to suggest that in the atg5 or atg7 mutant, the phagophore fails to generate a closed autophagosome. Please revisit and refine the model to accurately represent the observed findings and avoid potential misconceptions regarding the role of autophagy in phagophore development in the mutants.
Response: We have revised the model as you suggested. Please see the revised Fig. 6.

Minor point: Refining the entire manuscript, including figure legends, for both scientific and linguistic clarity would be helpful.
Response: We have carefully revised and refine the manuscript accordingly. In addition, we have improved the language of the manuscript with the help from a professional expert in the field.

References

1. Yan, H. *et al.* Autophagy and its mediated mitochondrial quality control maintain pollen tube growth and male fertility in Arabidopsis. *Autophagy* **19**, 768-783 (2023).
2. Wang, H. & Jiang, L. Transient expression and analysis of fluorescent reporter proteins in plant pollen tubes. *Nat Protoc* **6**, 419-426 (2011).

Response to reviewer 1

Reviewer #1 (Remarks to the Author):

This reviewer is not convinced that autophagy regulates the actin cytoskeleton in pollen via targeting and degrading ADF7 and Profilin2, as the authors stated that NBR1 depletion does not show obvious actin phenotype in pollen.

Response: Thank you very much for your constructive suggestions and comments on our manuscript. We have performed additional experiments using genetic, biochemical and cell biology approaches to further address your concerns from several lines of evidence:

i) We have performed additional genetic studies by generation of more genetic mutants including: *ADF*-RNAi*/atg5/atg7, Profilin*-RNAi*/atg5/atg7*, and *ADF*-RNAi*/Profilin*-RNAi*/atg5/atg7*. In addition to seed setting rates, we observed other defective phenotypes of the actin cytoskeleton associated with impaired autophagy. These phenotypes include abnormalities in sperm cell biogenesis within pollen grains, and the spatial organization of actin filaments in both pollen grains and pollen tubes in these various mutants. We conducted side-by-side comparisons of these phenotypes in both the WT and various mutants. Notably, the defective phenotypes of the actin cytoskeleton related to impaired autophagy were partially restored in *ADF*-RNAi*/atg5/atg7, Profilin*-RNAi*/atg5/atg7*, and *ADF*-RNAi*/Profilin*-RNAi*/atg5/atg7* when compared to *atg5/atg7*. These new results are included in **the revised Supplementary Fig. 11**. We also added and discussed the new results in the text of our manuscript, please see line 368-385 and 572-576.

ii) We assessed ADF and Profilin accumulation level in *nbr1-c2* and WT plants using western blotting and found no significant differences in the protein levels between *nbr1-2c* and the WT. This result is presented in **the revised Supplementary Fig.12b**.

iii) To better understand the lack of relevant *nbr1* phenotype and to investigate the potential involvement of other receptor(s) that might potentially function alongside NBR1, we conducted screening by luciferase complementation assays. We screened for alternative receptor(s) which may interact with ADF7 and Profilin2 based on known cytoplasmic autophagy receptors from previous studies[1-4]. We identified that the autophagic receptor family ATI can interact with both ADF7 and Profilin2. These findings are included in **the new Supplementary Fig.13**. The corresponding results and discussions have been added to our manuscript. Please see line 395-406 and 541-559.

In addition, this authors did not agree with the authors that characterization of the interaction of BBR1 with ADF7 and Profilin2 is beyond the scope of this study. This reviewer actually believes that it is an essential data to make this story publishable in the top-notched journal like Nature Communications.

Response: We have generated various domain truncations of NBR1 and systematically screened their interactions ADF7 and Profilin2 proteins by Y2H and luciferase complementation assays. Our results demonstrated that NBR1 binds to ADF7 and Profilin2 depending on both the ZZ-type zinc finger (ZZ) motif and the four-tryptophan (FW) domains. These new results are incorporated in the revised Fig. 5e-g. We also added the new results in the text of our manuscript, please see line 322-323 and 332-341.

Furthermore, this reviewer also has a minor concern about the description in Supplemental Figure 5, in which the authors simply grouped profilin as actin-depolymerizing factor might be inappropriate.
Response: We have made corrections. Please see line 276-278.

References

1. Zhou J, et al. Dicot-specific ATG8-interacting ATI3 proteins interact with conserved UBAC2 proteins and play critical roles in plant stress responses. Autophagy 14, 487-504 (2018).
2. Xie Q, et al. hfAIM: A reliable bioinformatics approach for in silico genome-wide identification of autophagy-associated Atg8-interacting motifs in various organisms. Autophagy 12, 876-887 (2016).
3. Marshall RS, Li F, Gemperline DC, Book AJ, Vierstra RD. Autophagic Degradation of the 26S Proteasome Is Mediated by the Dual ATG8/Ubiquitin Receptor RPN10 in Arabidopsis. Mol Cell 58, 1053-1066 (2015).
4. Wu J, Michaeli S, Picchianti L, Dagdas Y, Galili G, Peled-Zehavi H. ATI1 (ATG8-interacting protein 1) and ATI2 define a plant starvation-induced reticulophagy pathway and serve as MSBP1/MAPR5 cargo receptors. Autophagy 17, 3375-3388 (2021).