nature portfolio

Peer Review File

Stress Granules Sequester Autophagy Proteins to Facilitate Plant Recovery from Heat Stress

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Li and colleagues presents a novel regulatory mechanism of autophagy by the sequestration and release of different autophagy-related proteins into stress granules (SGs) specifically during heat stress and recovery respectively. This work reinforces recent notions of SG functioning beyond mRNA sequestration and presents the first report of plant SGs as regulators of autophagy, in contrast to the already reported role of autophagy in the clearance of SGs, reinforcing the tight interconnection and interdependency between both biological processes. Overall, the manuscript is well-written, concise and present enough experimental data to support most of the stated claims. However, there are several points that should be addressed.

Major points

• Figure 2a and Extended Data Figure 5: As none of the BiFC interactions in the control conditions were positive, it would be recommended to include a positive control (i.e. two proteins that are indeed present in the pre-interaction SG network, such as Rbp47b-YN/UBP1a-YC, to prove that BiFC occurs in such conditions and that the negative results obtained are indeed biologically relevant and not technical limitations.

• The protocol for isolation of SGs through differential centrifugations that the authors used is substantially different from the one cited (reference 17). As a rather new protocol, authors should describe it in more depth for reproducibility purposes: amount and nature of the starting sample, exact time, temperature and speed of every centrifugation step, the volume of the different resuspension steps (which is conflicting and/or unclear at some points), etc. Moreover, as the authors used their own version of the differential centrifugation isolation of SGs, they could use both markers (i.e. Rbp47b and UBP1) for both confocal observations (only mCherry-Rbp47b used) and immunoblot analyses (only UBP1a-FLAG used). Also, and as a related minor point, the “T”, “S” and “P” acronyms used throughout the manuscript figures, could be illustrated in Figure 2e to help the reader in identifying what each fraction represents.

• As it is the first case of such mutant in plants, the impact of the ubp1abc triple mutation in SG formation (i.e. monitoring mCherry-Rbp47b foci) should be done also in stable lines, not only in protoplasts. Only then, this could be compared to the results of the ATG13a, ATG6 and ATG5 puncta in Figure 3b-c.

• For the quantification of foci used in Figures 3c, 3e, 4b, 6b and S1f, as well as the co-localization analyses shown in Figures 1c, 5c and 5f, the authors need to include in the method section and/or figure legends the quantification procedures. Was it manual or in silico? Thresholds of intensity and/or size used? Software used for the quantification/co-localization? Number of independent experiments (that needs to be more than one) and sample size per experiment? Criteria to call for co-localization or not? The microscopy analyses section of the methods cannot only include the

parameters for the image acquisition, but should also include all the downstream processing as well when quantitative information is extracted out of it.

• Figure 6c: the nature of the precipitated fraction in the ubp1abc mutant should be described. According to Figure S1e-f (which was done in protoplasts), there are significantly less SGs in this background. Therefore, what is being pelleted in this background? Is it still SGs (therefore the need to immunoblot additional SG component such as Rbp47b as stated in the previous point)? Is it something else (actin is being partially pelleted in this fraction)? Why would ubiquitinated proteins cumulate specifically in these aggregates?

• Regarding the last point, authors claim that the role of the sequestration of certain ATG proteins into SGs during HS is to protect these proteins from thermal denaturation. I think there is not enough experimental data to validate this hypothesis. Authors clearly demonstrate that HS induces the relocation of certain ATG proteins to SGs during HS, and subsequent release thereof during recovery. They also prove how this release from the SG during recovery correlates with an increase in autophagic fluxes. However, whether this sequestration has a "protective" role on the ATG protein themselves is a mere hypothesis. Whether there is a protective role or if it is just another layer of post-translational regulation of the autophagic machinery, would need to be further studied. While this is not a major point of the manuscript, the parts where this hypothesis is mentioned (e.g. line 244, legend of Figure 6d…), should be dialed down and clearly state this is a possibility and not a experimentally validated statement.

Minor points

• Line 76: Authors state that a 38°C/1h treatment "mimics the high temperature conditions frequently encountered in nature". I cannot see how such condition would be frequently encountered in nature, nor how this would be of any relevance for the manuscript.

• Line 171: I think there is a mistake in the phrasing: "… suggesting the dependence of SG assembly for the translocation of ATG (not SG again) proteins to punctate structures during HS."

• Line 232: Authors state "Remarkably, substantial overlapping was observed between mCherry-ATG8f and NBR1-GFP on punctate structures after 3 h following HS recovery". Authors should perform a statistical test to quantitatively and unbiasedly claim whether the increase in co-localization during recovery is statistically significant or not.

• Figure 2b-d: The acronym "CK" was not explained neither in the figure legend nor the main text. I would suggest to rather write "control" as it is clearer and cohesive with the rest of the figures (unless CK conditions means something else?).

• Figure 3d-3: the authors comment on the lack of impact of the atg5-1 mutation in SG assembly during HS (lines 124-125), which is comparable to the WT. However, the authors also comment that the SG disassembly during the recovery phase is not affected neither by the atg5-1 mutation (lines 226-227 & 329-330). This does not seem to be the case, as the disassembly seems to be actually faster in the atg5-1 mutant (i.e. Rec. 3h of atg5-1 is similar to the Rec. 6h of WT). While this still indicates that autophagy is probably not required for the SG disassembly, the phrasing should better reflect the results obtained. Authors could rather say: "The disassembly of SGs is not prevented in the atg5-1 mutant". Additionally, they could also comment about why would the SG

recovery be faster in this background. Does this mutant copes differently with HS? What would be an hypothesis

• Figure 4a: The cytosolic signal of EYFP-ATG8f varies a lot at the different timepoints. Were all the images taken with the same settings? Otherwise, images with similar settings should be used instead to properly compare the images.

• Figure 4d: Quantitative experiments need to be conducted independently several times. While only one immunoblot can be maintained in the figure, the experiment should still be repeated two or three times, and the ratios shown in the figure illustrate the mean ± SEM from all experiments.

• Extended Data 1b-d: Images of full roots in Control conditions not shown. This should be included for comparisons, as done for Extended Data 1a and 1e, as well as main figures throughout the manuscript.

Reviewer #2 (Remarks to the Author):

The paper entitled 'Stress Granules Safeguard Autophagy Proteins to Facilitate Plant Recovery from Heat Stress' by Li and coworker is clear and concise. The study describes an original and novel process which brings critical understanding in the complex regulation of autophagy in plants.

The data presented mostly support the conclusion that a subset of ATG proteins are translocated in SGs upon HS, although whether the proteins are within autophagy compartments associated with SGs or genuinely in SGs remained to be proven by performing additional experiments (TEM). Notably, this conclusion is supported by the accumulation of ATG proteins alongside UPB1a in SGs in cell fractionation analyses (Fig.2e,g): in these conditions, the authors should show that the pellet is only composed of SGs and not additional cell compartments. Further, the authors do not provide substantial elements concerning the mechanism by which ATG proteins are recruited to SGs in these conditions. Although a subset of these proteins seems to directly interact with UBP1, how this interaction is triggered, regulated, disrupted upon HS recovery and how ATG proteins are still recruited in SGs in the absence of UPB1 is not tested, or discussed. Elucidating this mechanism (and the use of point mutants with disrupted ATG storage in SGs) would bring important information in the physiological role of ATG storage in SGs.

My main concern is about the physiological role of such translocation of ATG proteins. The authors propose that upon recovery re-translocation of ATG proteins in the cytoplasm facilitate a timely activation of autophagy to degrade HS-induced ubiquitinated aggregates. At this time, the manuscript does not provide data supporting this conclusion. The authors do not show that HS

induces ubiquitinated aggregates and they do not show that autophagy is induced upon HS recovery. Instead, the authors show in Fig.4 that priming with HS actually reduces autophagy activation by subsequent -C stress. This result is quite surprising but not properly addressed or discussed in the manuscript.

Results from Fig.4 and Fig.5 indicate a massive formation of ATG8f and NBR1 puncta after HS recovery suggesting an increase in autophagy but proper experiments need to be performed to reach this conclusion. Authors should follow GFP-ATG8 cleavage before HS, after HS and after HS recovery in rich conditions. If the authors hypothesis is correct, autophagy flux should be induced and free GFP should accumulate. Further, in Fig.4a,b, authors should include the use of concanamycin A in the same conditions to monitor not only the formation of ATG8/NBR1 puncta but also their accumulation in the vacuole to properly monitor autophagy flux. It is to be noted that the authors provide data resulting from the use of ConcA (recovery after 12h) but with lack of proper controls (same analyses before HS, upon HS and after several time of recovery) it is complicated to conclude whether autophagy has been induced. These analyses should also be performed in the ubp triple mutant (to complete Fig.6) to determine how impairment of ATG storage in SGs affect autophagy activity/flux. Notably, in Fig.6c: authors suggest that there is more ubiquitinated proteins in the ubp1abc mutant: this is not obvious to me, the authors need to provide a quantification of these experiments with additional independent replicates and statistical analyses. Further, the authors propose that HS induce the ubiquitinated aggregates and that autophagy is involved in their clearance, more efficiently because ATG proteins are stored in SGs in these conditions. The authors should provide data that support this hypothesis. Specifically, the authors should show how HS affects the level of ubiquitinated proteins (1h 38°C) and then follow the level of ubiquitinated proteins upon a time course of recovery +/- formation of SGs (ubp1abc mutant) or autophagy (atg mutant).

Specific comments:

- In extended data Fig.4, analyses of ATG18 do show some colocalization. Why use another marker of SGs? (UBP1c for all expect for ATG18: RBP47?)

- Fig. 2b-d, please define CK

- Fig. 2g, please provide quantification of at least 3 independent experiments

- Fig.3d,e: why is the disappearance of UBP1c SGs is much more rapid in atg5-1 than in WT in recovery experiments? Please provide quantification and discussion of this result.

- Fig.4c, left panel: the authors mention finding ATG8f on membraneless structure. From the data provided I disagree: ATG8f particles seem to decorate a cup-shape structure resembling the initial

autophagy compartment (isolation membrane, phagophore). The authors need to provide additional images. These will provide important information on the nature of the ATG8f puncta that massively accumulate as soon as after 1h of recovery.

Fig.4d, need quantification of 3 independent experiments. What is MS-C medium: solid plates? Liquid? What about 'untreated' plants? Were they still put in liquid medium etc? Why would autophagy be reduced in plants primed for HS? It seems to go in the opposite direction of the authors conclusion that translocation of ATG proteins to SGs facilitates the activation of autophagy.

- How is heat stress treatment performed/controlled? Are the unstressed plants also put in liquid medium in a water bath at 22C as control? What about light conditions during HS?

- From Fig. 4 the authors conclude that: (line 207-208) ' These findings imply that the autophagy pathway is inhibited during the HS phase and the early stage of recovery, whereas it is activated in the later stage of HS recovery.' Results presented in that figure do not support this conclusion, in my opinion.

(1) The massive accumulation of ATG8 puncta suggest a large induction of autophagy as soon as 1h of recovery and peaking at 6 h of recovery (Fig. 4a); (2) from the one picture shown in Fig.4c left panel, ATG8f is found on isolation membrane/phagophore in these conditions (showing at least start of autophagosome formation); (3) HS-primed plants show reduced GFP-ATG78f degradation compared to untreated plants upon -C conditions suggesting lower autophagy flux.

Based on (1) and (2), we can suppose that autophagy is induced quite rapidly after HS recovery. This has to be experimentally tested by performing GFP-ATG8 degradation assays upon HS and after a time course of recovery in rich conditions; additionally, using concanamycin A in the same conditions as performed in Fig. 4a,b, should inform on the amount of autophagosomes formed and translocated in the vacuole upon each of these conditions/time (again, to follow flux).

Based on (3), it seems that HS-primed plants actually have lower autophagy flux than untreated plants: why would the authors then conclude that (lines 215-216): 'release of ATG proteins from disassembled SGs aids in initiating the activation of autophagy during the HS recovery phase'. I am not sure to follow the rational here. This conclusion is in clear contradiction with results that are presented in Fig.4d. The authors should properly discuss this result and explain why they tested -C induced autophagy after HS recovery? Again, the authors should assess autophagy flux using the same assay but looking at before HS, after HS and in a time course of recovery, compared to plants put in the same conditions without HS.

- Fig. 5

Autophagy involved in disassembly of SGs (after HS) in mammals. No colocalization between ATG8f and SGs. What about other ATG8s? Other components of the autophagy machinery that are more

ubiquitous than ATG8 were multiple isoforms could be involved in dedicated types of autophagy? ATG18 for instance.

- Line 226: 'Disassembly of SGs is not autophagy dependent' (Fig.3d,e): actually the disassembly appears much faster in the atg5-1 mutant: why ?

- Fig.5b, what is control: HS-treated plants? Untreated plants? Why is NBR1 forming many puncta in untreated conditions (or in HS conditions): is there also an accumulation of NBR1 in SGs? At 3h: many co-localizations: seem to indicate that autophagy is already induced. Why not look at concA in these conditions rather than after 12h of recovery? Fig. 5e lacks proper control (no priming) and without concA.

- Lines 244-245: 'The above results suggest that SGs may potentially safeguard ATG proteins, whose release during the HS recovery phase facilitates the rapid activation of autophagy for cell survival.' The above results or conclusions from the authors actually suggest that HS somewhat reduces autophagy (when seedlings are primed with HS, they induce less autophagy).

- Fig.6a,b is not a direct measurement of autophagy. The authors need to look at flux rather than only number of ATG8 structures. There may be less structures because more autophagosomes already translocated in the vacuole. Again, the authors need to use concA to assess this.

- Fig.6c: the authors suggest that there is more ubiquitinated proteins in the ubp1abc mutant: this is not obvious to me, the authors need to provide a quantification of these experiments with additional independent replicates and statistical analyses. Further, the authors propose that HS induce the ubiquitinated aggregates and that autophagy is involved in their clearance, more efficiently because ATG proteins are stored in SGs in these conditions. The authors should provide data that support this hypothesis. Specifically, the authors should show how HS affects the level of ubiquitinated proteins (1h 38°C) and then follow the level of ubiquitinated proteins upon a time course of recovery +/- formation of SGs (ubp1abc mutant) or autophagy (atg mutant).

- The authors should also provide more elements, at least in the discussion, about the potential molecular mechanisms by which these particular ATG proteins are recruited and stored in SGs. For instance, the authors show a direct interaction between UBP1a and several ATG proteins, specifically during HS. By exploring the sequences/3D structure of SGs-stored ATG proteins, could the authors identify a specific domain that binds to UBP1a? How is this interaction regulated upon HS? In the absence of either of the 3 UBP1 proteins, the association of ATG proteins with SGs is significantly reduced but about a third of the puncta are still formed: this implies that UPB1 is not solely responsible for ATG interactions with SGs. How many SGs are still formed in the ubp1abc triple mutant in the same conditions? The authors provide part of the answer in Fig.S1, by looking at

RBP47B in protoplasts of the ubp1abc mutant after 30 min of HS. Unfortunately, the image shown in Fig.S1 could be of better quality, further, for consistency and proper conclusion, HS should be performed after 1h in roots of Arabidopsis.

- The authors discuss about the different environmental signals leading to different populations of SGs. Similarly, different stresses/signals cause different types of autophagy, notably though the recruitment of specific cargo and the use of specific ATG8 proteins. In this paper, the authors solely based their autophagy measurement on ATG8f. Is there a particular reason for that? What about other ATG8s? Are they stored in SGs? Are they involved in activating autophagy upon HS stress or HS stress release?

Previous analyses showed that autophagy is activated upon HS, how does that compare to the mechanism/process that the authors are presenting here? If ATG proteins are stored in SGs how can they participate in autophagy induction during HS? Are ATG proteins stored only transiently in these SGs? Why?

Reviewer #3 (Remarks to the Author):

Li et al. demonstrate that during mild heat shock (HS) conditions, essential autophagy-related (ATG) proteins relocate to stress granules (SGs), resulting in a temporary suppression of autophagy. Following the alleviation of HS, these ATG proteins are released into the cytoplasm, aiding in the removal of ubiquitinated proteins. While the study is interesting, some of its conclusions lack sufficient supporting data. Several points are needed to be addressed to support their conclusions more firmly.

Major comments:

1-The study applies a mild heat shock (HS) treatment of 38°C for 1 hour often referred to as priming HS (also in Fig.4d). Yet, the formation of SGs and the activation of autophagy can be triggered by a wide range of HS intensities. To clarify and strengthen their findings, the authors should determine whether the observed effects are unique to mild HS conditions or if they apply broadly to different HS intensities, including severe HS scenarios cited in their other work. Should these effects be specific to mild HS, the authors are encouraged to explore or explain the biological importance and regulatory mechanisms of this specificity.

2-In Figure 3b/c, the authors demonstrate a significant decrease in puncta of ATG13a, ATG6, and ATG5 GFP fusions in the ubp1abc mutant compared to WT plants following 1h of HS, highlighting SG assembly's role in the translocation of ATG proteins to SGs. However, to support this conclusion, it's crucial to ensure that the observed differences aren't due to lower baseline expression or protein levels of ATGs in the mutant. The authors should present the basal expression and abundance of these ATGs in both Col-0 and the ubp1abc mutant using both qRT-PCR and western blotting.

3-Figure 3d presents representative images illustrating the gradual decrease in the number of stress granules (SGs) during recovery from heat shock (HS); however, the data lacks quantification, and this should be provided. Furthermore, although there is no difference in the formation of SGs puncta upon HS treatment, the number of SGs significantly decreased or (nearly absent) in the atg5-1 background at 3h after recovery. This observation suggests a crucial role of ATG proteins, including ATG5, in the maintenance and persistence of SGs beyond their initial formation. Additional comments on this observation are recommended.

4-Extended Data Fig. 1e: The authors did not use concanamycin A (as otherwise, it would have been mentioned in the legend or methods). Without ConA, the rare puncta formation is likely observed due to autophagosome degradation from high acidic vacuolar PH. To ensure the conclusion of the absence of autophagosomes during mild HS (in both Extended Data Fig. 1e and Figure 4a), the authors should repeat the experiment by including ConA and appropriate controls.

5-Figure 6a/b: the authors discuss that circular autophagosomes labeled by EYFP-ATG8f were delayed in ubp1abc mutant in comparison to WT plants However, since ConA wasn't used, it's unclear if these puncta are truly autophagosomes or any other types of condensates/aggregates. I am not aware of any reports showing such massive autophagosome accumulation without the use of ConA. Additionally, to confirm the delayed autophagic activity, further experiments beyond microscopy, such as autophagic flux assays and immunoblotting for NBR1 and ATG8 levels, are necessary.

Furthermore, the authors should examine and discuss any potential differences in autophagic activity between EGFP-ATG8f/ubp1abc mutant and EGFP-ATG8f under control conditions. This analysis could influence the interpretation of the results presented in Figure 6a/b.

6- Figure 4a, again, the authors did not use ConA (see the comment above).

7-Figure 4d. Surprisingly, higher autophagic activity is detected under control conditions at 6 and 12 hours (EYFP/EYFP-ATG8f ratios of 1.15 and 3.59) compared to post-heat treatment (EYFP/EYFP-ATG8f ratios of 0.06 and 1.17). The authors should discuss potential reasons for this unexpected finding and include control images for autophagosome detection, as in Figure 4a, for better clarity.

While EYFP/EYFP-ATG8f effectively indicates autophagic activity, supporting this data with additional metrics is crucial, especially by assessing NBR1 and ATG8 levels through immunoblotting, for which reliable antibodies are available at Abcam.

8-In lines 238-240, the author notes the role of NBR1 in releasing autophagic activity to eliminate proteins marked by NBR1 during recovery from heat stress (HS). To further validate this claim, measuring NBR1 levels in the ubp1abc mutant during HS and recovery is essential, as they serve as an indicator of autophagic activity and its participation in this process.

Minor comments:

1) The use of UBP1a for BiFC and Co-IP experiments (Figure 2a,b) and UBP1c for co-localization studies (Figure 1b) raises questions about the rationale behind choosing different UBP1 isoforms for these experiments. Given that UBP1a, UBP1b, and UBP1c are distinct proteins located at the core of stress granules in Arabidopsis thaliana, it would be helpful if the authors could clarify why specific isoforms were selected for particular assays.

2) Line 287 and Fig 3a: “These ATG puncta were induced by HS, but not by sodium arsenite (Fig. 3a), indicating the specific involvement of HS-induced SGs in the regulation of autophagic activity, but not other stress-induced SGs”. This is a very strong generalized statement regarding other abiotic stresses.

Recent studies have demonstrated the induction of autophagy and SG formation by various stressors, such as carbon starvation, oxidative stress, salt, drought, and cold. It's likely that proteins such as ATG5, ATG6, and ATG13 also translocate to SGs under other stress conditions. Further experiments are needed to provide evidence for this hypothesis. I, therefore, recommend authors moderate the tone.

3) In Figure 1c, "n=10" needs clarification: does it refer to 10 roots, 10 cells, or something else? Additionally, the analysis should specify the size of the root area studied for co-localization

4) Co-localization data shown in Figure 2a, Extended data figures 2a,b and 3a,b should be quantified.

5) Extended data Fig. 1b- Please include the control images for roots without heat stress.

6) Line 140-141- The reduction in the levels of ATG proteins and UBP1a-FLAG in the supernatant should be quantified.

7) Figure 6C- It is important that authors provide quantitative analyses to better present/clarify the differences in the abundance of ubiquitinated proteins between ubp1abc mutants and wild-type (WT).

8) Lines 154-158—Heat stress is often accompanied by oxidative stress. I recommend to discuss why arsenite treatment, which causes oxidative stress, cannot change the localization of ATG proteins.

9) Line 224- colocation should be colocalization.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Responses to reviewers' comments

Reviewer #1 (Remarks to the Author):

The manuscript by Li and colleagues presents a novel regulatory mechanism of autophagy by the sequestration and release of different autophagy-related proteins into stress granules (SGs) specifically during heat stress and recovery respectively. This work reinforces recent notions of SG functioning beyond mRNA sequestration and presents the first report of plant SGs as regulators of autophagy, in contrast to the already reported role of autophagy in the clearance of SGs, reinforcing the tight interconnection and interdependency between both biological processes. Overall, the manuscript is well-written, concise and present enough experimental data to support most of the stated claims. However, there are several points that should be addressed.

Response: We appreciate your constructive feedback and your time in carefully reviewing our manuscript. We have thoroughly considered your comments and made significant revisions accordingly.

Major points

• Figure 2a and Extended Data Figure 5: As none of the BiFC interactions in the control conditions were positive, it would be recommended to include a positive control (i.e. two proteins that are indeed present in the pre-interaction SG network, such as Rbp47b-YN/UBP1a-YC, to prove that BiFC occurs in such conditions and that the negative results obtained are indeed biologically relevant and not technical limitations.

Response: Thank you for your suggestion. We have now included Rbp47b and UBP1a as a positive pair and performed BiFC assay to confirm their association in the pre-interaction SG network under normal growth conditions. The new results are shown in **Extended Data Fig. 3a** with appropriate description in the revised manuscript (**Page 6, highlighted in magenta**).

• The protocol for isolation of SGs through differential centrifugations that the authors used is substantially different from the one cited (reference 17). As a rather new protocol, authors should describe it in more depth for reproducibility purposes: amount and nature of the starting sample, exact time, temperature and speed of every centrifugation step, the volume of the different resuspension

steps (which is conflicting and/or unclear at some points), etc. Moreover, as the authors used their own version of the differential centrifugation isolation of SGs, they could use both markers (i.e. Rbp47b and UBP1) for both confocal observations (only mCherry-Rbp47b used) and immunoblot analyses (only UBP1a-FLAG used). Also, and as a related minor point, the “T”, “S” and “P” acronyms used throughout the manuscript figures, could be illustrated in Figure 2e to help the reader in identifying what each fraction represents.

Response: Thank you very much for the valuable suggestion. We have now provided detailed procedures of this method in the Materials and Methods section (**Page 19-20, highlighted in magenta**). Following your suggestion, we have now employed this method to isolate SGs from cell with co-expression of mCherry-Rbp47b and UBP1a-GFP, and verified their co-localization on the isolated SGs. The new results are presented in **Fig. 2f** with appropriate description (**Page 6, highlighted in magenta**). We have also labeled the “T”, “S” and “P” acronyms in **Fig 2e**.

• As it is the first case of such mutant in plants, the impact of the *ubp1abc* triple mutation in SG formation (i.e. monitoring mCherry-Rbp47b foci) should be done also in stable lines, not only in protoplasts. Only then, this could be compared to the results of the ATG13a, ATG6 and ATG5 puncta in Figure 3b-c.

Response: Thanks for your suggestion. We have now obtained transgenic plants stably expressing mCherry-RBP47b in WT and *ubp1abc* mutant, and have confirmed the reduction of HS-induced mCherry-Rbp47b foci in *ubp1abc* mutant than the WT seedlings. The new results are shown in **Supplementary Fig. 3 e-g**.

• For the quantification of foci used in Figures 3c, 3e, 4b, 6b and S1f, as well as the co-localization analyses shown in Figures 1c, 5c and 5f, the authors need to include in the method section and/or figure legends the quantification procedures. Was it manual or in silico? Thresholds of intensity and/or size used? Software used for the quantification/co-localization? Number of independent experiments (that needs to be more than one) and sample size per experiment? Criteria to call for co-localization or not? The microscopy analyses section of the methods cannot only include the parameters for the image acquisition, but should also include all the downstream processing as well when quantitative information is extracted out of it.

Response: Thanks for your suggestion. We employed ImageJ program with

various plugins to identify the puncta, count their numbers and perform co-localization analyses. The detailed method has now been included in the revised manuscript (**Page 20-21, highlighted in magenta**).

• Figure 6c: the nature of the precipitated fraction in the *ubp1abc* mutant should be described. According to Figure S1e-f (which was done in protoplasts), there are significantly less SGs in this background. Therefore, what is being pelleted in this background? Is it still SGs (therefore the need to immunoblot additional SG component such as Rbp47b as stated in the previous point)? Is it something else (actin is being partially pelleted in this fraction)? Why would ubiquitinated proteins cumulate specifically in these aggregates?

Response: Thank you very much for your comments. In Fig 6c (**now Fig 6f**), our objective is to detect ubiquitinated insoluble protein aggregates rather than SGs. According to previous reports, sequestering ubiquitinated insoluble and potentially cytotoxic proteins into aggregates is crucial for maintaining cellular homeostasis. These aggregates often exceed the size threshold for proteasomal degradation and are predominantly degraded by the autophagy pathway. In plants, NBR1 has been identified as an autophagy receptor, which binds to the K63-linked ubiquitin chains to facilitate the targeting of protein aggregates into autophagy pathway followed by degradation inside vacuoles. Therefore, the presence of ubiquitinated insoluble proteins, as shown in **new Fig 6f**, is indicative of the efficiency of autophagy-mediated degradation of these protein aggregates.

SGs and protein aggregates are both described as membrane-less particles in the cytoplasm. However, SGs are characterized as functional protein-RNA complexes, whereas protein aggregates consist of a large amount of denatured proteins. Previous studies have shown that these two types of particles can be separated by differential centrifugation. Consequently, both types of particles may coexist in the pellet after HS 1h (**new Fig 6f**). We acknowledge that ubiquitination also occurs within SGs, complicating the distinction between SGs and protein aggregates. Nonetheless, by 6 to 12 hours post-HS recovery, SGs have completely disassembled (as shown in **new Figure 3d, e**), whereas ubiquitinated insoluble proteins remain detectable. This persistence suggests that ubiquitin antibodies are a reliable tool for the detection of protein aggregates at later stage (6-12 h) of HS recovery.

• Regarding the last point, authors claim that the role of the sequestration of certain ATG proteins into SGs during HS is to protect these proteins from thermal denaturation. I think there is not enough experimental data to validate this hypothesis. Authors clearly demonstrate that HS induces the relocation of certain ATG proteins to SGs during HS, and subsequent release thereof during recovery. They also prove how this release from the SG during recovery correlates with an increase in autophagic fluxes. However, whether this sequestration has a "protective" role on the ATG protein themselves is a mere hypothesis. Whether there is a protective role or if it is just another layer of post-translational regulation of the autophagic machinery, would need to be further studied. While this is not a major point of the manuscript, the parts where this hypothesis is mentioned (e.g. line 244, legend of Figure 6d…), should be dialed down and clearly state this is a possibility and not a experimentally validated statement.

Response: Thanks for your suggestion. We agree with your opinion and have rephrased the title and text to tone down the description about the protective role of SGs on ATG proteins. The possible roles of SGs on sequestering ATG proteins have been discussed (**Page 14-15, highlighted in magenta**).

Minor points

• Line 76: Authors state that a 38°C/1h treatment "mimics the high temperature conditions frequently encountered in nature". I cannot see how such condition would be frequently encountered in nature, nor how this would be of any relevance for the manuscript.

Response: Thanks for your suggestion. We have corrected this inappropriate description.

• Line 171: I think there is a mistake in the phrasing: "… suggesting the dependence of SG assembly for the translocation of ATG (not SG again) proteins to punctate structures during HS."

Response: We have made corrections.

• Line 232: Authors state "Remarkably, substantial overlapping was observed between mCherry-ATG8f and NBR1-GFP on punctate structures after 3 h following HS recovery". Authors should perform a statistical test to quantitatively and unbiasedly claim whether the increase in co-localization during recovery is statistically significant or not.

Response: We have performed a statistical analysis of the co-localization ratio between mCherry-ATG8f foci and NBR1-GFP puncta and found that their co-localization ratio was significantly increased following recovery from HS (**Fig. 5c**).

• Figure 2b-d: The acronym “CK” was not explained neither in the figure legend nor the main text. I would suggest to rather write “control” as it is clearer and cohesive with the rest of the figures (unless CK conditions means something else?).
Response: We have replaced "CK" with "control" throughout the manuscript.

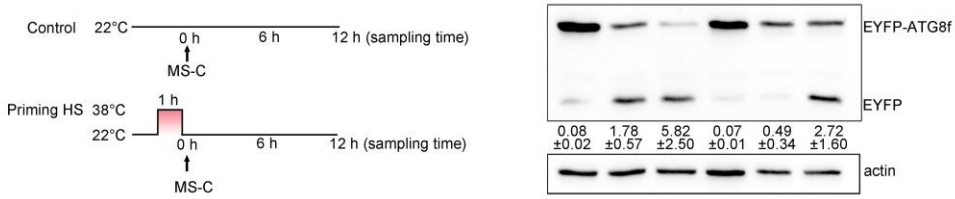
• Figure 3d-3: the authors comment on the lack of impact of the atg5-1 mutation in SG assembly during HS (lines 124-125), which is comparable to the WT. However, the authors also comment that the SG disassembly during the recovery phase is not affected neither by the atg5-1 mutation (lines 226-227 & 329-330). This does not seem to be the case, as the disassembly seems to be actually faster in the atg5-1 mutant (i.e. Rec. 3h of atg5-1 is similar to the Rec. 6h of WT). While this still indicates that autophagy is probably not required for the SG disassembly, the phrasing should better reflect the results obtained. Authors could rather say: “The disassembly of SGs is not prevented in the atg5-1 mutant”. Additionally, they could also comment about why would the SG recovery be faster in this background. Does this mutant copes differently with HS? What would be an hypothesis
Response: Thanks for your comments. We are very sorry for our mistake. There was an error in the labeling of images in the *atg5-1* background, where it was incorrectly indicated as a '3-hour recovery' when it should have been '6-hour recovery'. This mistake has been corrected in the revised manuscript. In addition, we have provided detailed statistical results on the number of SG in WT and *atg5-1* mutants (**new Fig. 3e**).

• Figure 4a: The cytosolic signal of EYFP-ATG8f varies a lot at the different timepoints. Were all the images taken with the same settings? Otherwise, images with similar settings should be used instead to properly compare the images.
Response: Thank you for your comments. We have reperformed confocal observation by using the same settings to ensure consistency and to normalize for any background noise. The updated images are now presented in **Fig. 4a**.

• Figure 4d: Quantitative experiments need to be conducted independently several times. While only one immunoblot can be maintained in the figure, the experiment should still be repeated two or three times, and the ratios shown in the figure illustrate the mean ± SEM from all experiments.

Response: Thanks for your comments. In the original Figure 4d, we had implemented a carbon starvation treatment subsequent to heat stress to illustrate the inhibition of the autophagy pathway during the heat stress phase and the early recovery stage, with subsequent activation during the later stages of recovery. We agree with your valuable insight and that of other reviewers that introducing an additional stressor, such as carbon starvation, complicates the interpretation of the results. After careful deliberation, we have decided to remove this result (Fig. 4d in previous manuscript). Instead, we replaced this experiment with concanamycin A treatment to demonstrate the accumulation of autophagic bodies inside vacuoles after HS (**new Fig. 4d**). We also found that the HS-primed plants exhibited an enhanced accumulation of autophagic bodies inside vacuoles compared to the untreated controls (**new Fig 5e and 5f**).

Fig. 4 d in Previous manuscript



Original Fig 4d. The 5-day-old EYFP-ATG8f plants were first subjected to treatment at 38°C or left untreated, and then transferred to MS-C medium to induce autophagy. Samples were collected at the indicated time points followed by immunoblotting analysis. The values represent the ratio of EYFP to EYFP-ATG8.

• Extended Data 1b-d: Images of full roots in Control conditions not shown. This should be included for comparisons, as done for Extended Data 1a and 1e, as well as main figures throughout the manuscript.

Response: We have now added the confocal images of full roots in control conditions (**new Extended Data Fig 1 b-d**).

Reviewer #2 (Remarks to the Author):

The paper entitled 'Stress Granules Safeguard Autophagy Proteins to Facilitate Plant Recovery from Heat Stress' by Li and coworker is clear and concise. The study describes an original and novel process which brings critical understanding in the complex regulation of autophagy in plants.

Response: We appreciate your time in carefully reviewing our manuscript and your constructive comments. We have thoroughly considered your comments and made significant revisions accordingly.

The data presented mostly support the conclusion that a subset of ATG proteins are translocated in SGs upon HS, although whether the proteins are within autophagy compartments associated with SGs or genuinely in SGs remained to be proven by performing additional experiments (TEM). Notably, this conclusion is supported by the accumulation of ATG proteins alongside UPB1a in SGs in cell fractionation analyses (Fig.2e,g): in these conditions, the authors should show that the pellet is only composed of SGs and not additional cell compartments. Further, the authors do not provide substantial elements concerning the mechanism by which ATG proteins are recruited to SGs in these conditions. Although a subset of these proteins seems to directly interact with UBP1, how this interaction is triggered, regulated, disrupted upon HS recovery and how ATG proteins are still recruited in SGs in the absence of UPB1 is not tested, or discussed. Elucidating this mechanism (and the use of point mutants with disrupted ATG storage in SGs) would bring important information in the physiological role of ATG storage in SGs.

Response: Thank you very much for your constructive comments. To further examine the subcellular localization of ATG proteins, we have performed the immuno-TEM (transmission electron microscopy) analysis using GFP antibodies on ultrathin sections prepared from high-pressure frozen/freeze-substituted roots of HS-treated ATG13a-GFP plants. The TEM imaging results revealed that the gold particles were frequently concentrated in electron-dense areas with irregular shapes, whose morphology resembles that of SGs shown in previous reports. These new results are shown in **Figure 1d** with appropriate description in the revised manuscript (**Page 5, highlighted in magenta**).

In this manuscript, we utilized differential centrifugation as a method for isolation of SG-enriched cellular fraction. We have not conducted negative staining electron microscopy to assess the purity of the SGs particles within the

pellets. Therefore, we cannot exclude the presence of other cellular compartments, such as large protein aggregates or ribosomes, within the SGs-enriched pellets. Although our approach may not perfectly isolate pellets composed exclusively of SGs, it has proven to be a simple and effective means of obtaining SG-enriched fractions. This is supported by the detection of two distinct SG markers, RBP47b and UBP1c, within the pellets as shown in **Fig 2f**. Based on these findings, we believe that our method is suitable for isolating SG-enriched fractions and for monitoring the distribution of SG components and ATG proteins in response to HS.

Furthermore, we have discussed the potential mechanisms of ATGs recruitment to SGs (**page 12-13, highlighted in magenta**). A scaffold-client working model has been employed to elucidate the role of core components (scaffold proteins essential for SGs formation) and client components (biomolecules recruited via scaffold-client interactions) in SGs formation as described in previous studies. In this model, SG assembly is driven by oligomerization and phase-separation of scaffold proteins to from a scaffold-scaffold-RNA bond network. Subsequently, client proteins are recruited to scaffold-scaffold binding sites by interaction with scaffold. In this study, we demonstrated that ATG proteins might not contribute to the driving force of SG assembly (Fig. 3d and 3e), suggesting that they act as client proteins within SGs. Given that the *ubp1abc* triple mutant showed an obvious defect in the formation of HS-induced SGs, as indicated by the core component mCherry-Rbp47b (**Supplementary Fig. 3e**), the reduction in ATGs-labeled puncta in *ubp1abc* mutant might be attributed to the defect in SGs assembly. Therefore, despite observing an interaction between ATG proteins and components of SGs under HS conditions in *vivo* (**Fig. 2**), the precise scaffold components that are directly responsible for recruiting ATG proteins have yet to be identified.

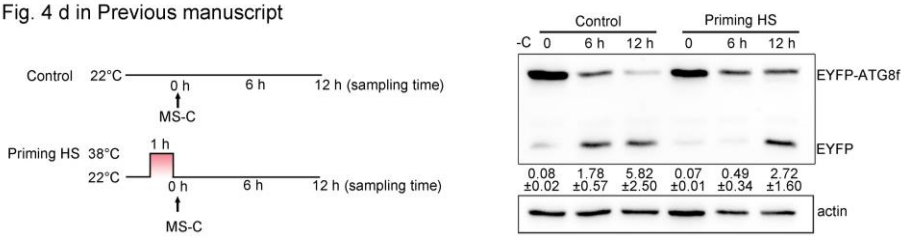
My main concern is about the physiological role of such translocation of ATG proteins. The authors propose that upon recovery re-translocation of ATG proteins in the cytoplasm facilitate a timely activation of autophagy to degrade HS-induced ubiquitinated aggregates. At this time, the manuscript does not provide data supporting this conclusion. The authors do not show that HS induces ubiquitinated aggregates and they do not show that autophagy is induced upon HS recovery. Instead, the authors show in Fig.4 that priming with HS actually reduces autophagy activation by subsequent -C stress. This result is quite surprising but not properly addressed or discussed in the manuscript.

Response: Thanks for your valuable suggestion. Following your suggestion, we have now examined the abundance of ubiquitinated insoluble proteins in the WT and *ubp1abc* mutants during the HS and recovery phases (new **Fig. 6f-g**). The obtained results demonstrates that the ubiquitinated insoluble protein increased markedly after HS and did not decrease significantly within the first 6 h of recovery, but showed a significant reduction after 12 h of recovery. This aligns with the appearance of circular autophagosomes and the accumulation of autophagic bodies at 9-12 h post-HS recovery (**Fig. 4**). Notably, the results showed that the enrichment of ubiquitinated insoluble proteins was significantly higher in *ubp1abc* mutants than the WT plants at 12 h following HS recovery (new **Fig. 6f-g**), coinciding with the observation of reduced autophagy activity in the *ubp1abc* mutant during the HS recovery phase (new **Fig. 6c, 6d**).

In addition, to further assess the autophagy activity after HS, we treated HS-primed plants with Concanamycin A (Conc A) to visualize the autophagic bodies inside vacuoles. The obtained results demonstrated that the EYFP-ATG8f-labeled autophagic bodies inside vacuoles were barely detectable during the first 3 h of recovery, with only a few observed at 6 h post-recovery. However, a significant number of autophagic bodies inside vacuoles could be clearly observed at 9 and 12 h post-recovery (**Fig. 4d, e**). This is consistent with the observation of reduced levels of ubiquitinated proteins at 12 h post-recovery from HS.

In the original Figure 4d, we had implemented a carbon starvation treatment subsequent to heat stress to illustrate the inhibition of the autophagy pathway during the heat stress phase and the early recovery stage, with subsequent activation during the later stages of recovery. We agree with your valuable insight and that of other reviewers that introducing an additional stressor, such as carbon starvation, complicates the interpretation of the results. After careful deliberation, we have decided to remove this result (Fig. 4d in previous manuscript). Instead, we replaced this experiment with concanamycin A treatment to demonstrate the accumulation of autophagic bodies inside vacuoles after HS. We found that the HS-primed plants exhibited an enhanced accumulation of autophagic bodies inside vacuoles compared to the untreated controls (**new Fig 5e and 5f**). These new data are now presented in the **Figures 4d, 4e, 5e and 5f** with appropriate description in the text (**Page 9-11, highlighted in magenta**) to ensure clarity and coherence with our revised conclusions.

Fig. 4 d in Previous manuscript



Original Fig 4d. The 5-day-old EYFP-ATG8f plants were first subjected to treatment at 38°C or left untreated, and then transferred to MS-C medium to induce autophagy. Samples were collected at the indicated time points followed by immunoblotting analysis. The values represent the ratio of EYFP to EYFP-ATG8.

Results from Fig.4 and Fig.5 indicate a massive formation of ATG8f and NBR1 puncta after HS recovery suggesting an increase in autophagy but proper experiments need to be performed to reach this conclusion. Authors should follow GFP-ATG8 cleavage before HS, after HS and after HS recovery in rich conditions. If the authors hypothesis is correct, autophagy flux should be induced and free GFP should accumulate. Further, in Fig.4a,b, authors should include the use of concanamycin A in the same conditions to monitor not only the formation of ATG8/NBR1 puncta but also their accumulation in the vacuole to properly monitor autophagy flux. It is to be noted that the authors provide data resulting from the use of ConcA (recovery after 12h) but with lack of proper controls (same analyses before HS, upon HS and after several time of recovery) it is complicated to conclude whether autophagy has been induced. These analyses should also be performed in the ubp triple mutant (to complete Fig.6) to determine how impairment of ATG storage in SGs affect autophagy activity/flux. Notably, in Fig.6c: authors suggest that there is more ubiquitinated proteins in the ubp1abc mutant: this is not obvious to me, the authors need to provide a quantification of these experiments with additional independent replicates and statistical analyses. Further, the authors propose that HS induce the ubiquitinated aggregates and that autophagy is involved in their clearance, more efficiently because ATG proteins are stored in SGs in these conditions. The authors should provide data that support this hypothesis. Specifically, the authors should show how HS affects the level of ubiquitinated proteins (1h 38°C) and then follow the level of ubiquitinated proteins upon a time course of recovery +/- formation of SGs (ubp1abc mutant) or autophagy (atg mutant).

Response: Thank you very much for your constructive suggestion. We have tried several times to conduct GFP-ATG8 cleavage assay to quantify the changes in autophagy flux before and after HS. Unfortunately, we were not able to reach a consistent conclusion, because we found that it was hard to detect

the free GFP protein bands cleaved from GFP-ATG8 during the 12 h recovery period after HS. This challenge arose when the HS-primed plants were put in the standard 1/2 MS medium with 1% sucrose for recovery. Therefore, following your suggestions, we shifted our approach to apply concanamycin A treatment to HS-primed plants, which allowed us to observe the accumulation of autophagic bodies inside vacuoles during the recovery from HS (**Fig. 4d**).

Furthermore, we have conducted concanamycin A treatment on plants co-expressing NBR1-GFP and mCherry-ATG8f. This experiment revealed an enhanced accumulation of NBR1/ATG8 bodies inside the vacuoles of HS-primed plants compared to the plants without HS. This result is shown in the new **Figures 5e and 5f** with appropriate description in the text (**Page 11, highlighted in magenta**). Additionally, we applied concanamycin A during the recovery phase to both EYFP-ATG8f/WT and EYFP-ATG8f/*ubp1abc* mutant plants. The obtained results revealed a significant reduction in the accumulation of EYFP-ATG8f-labeled autophagic bodies in the *ubp1abc* mutant compared to WT plants at 9 and 12 hours post-HS recovery, suggesting a delay in autophagy activation in the *ubp1abc* mutant during the recovery phase, as illustrated in **Figure 6c and 6d** with appropriate description in the text (**Page 11, highlighted in magenta**).

To reinforce our findings, we have also performed immunoblotting with anti-ATG8 antibodies to assess the lipidated and non-lipidated forms of ATG8. The lipidated ATG8 (ATG8-PE adduct) mainly labels the forming autophagic vesicles and completed autophagosomes, and the ratio of ATG8–PE to ATG8 is indicative of autophagy activity. The newly obtained results demonstrated that the ATG8-PE adduct appeared during the post-HS recovery period, but the ATG8-PE/ATG8 ratio was considerably lower in the *ubp1abc* mutant than in WT plants (**new Extended Data Fig. 12**), indicating reduced autophagy activity in the mutant.

Lastly, we have examined the abundance of ubiquitinated insoluble proteins in the WT and *ubp1abc* mutants during the HS and recovery phases (**Fig. 6f-g**). The ubiquitinated insoluble protein increased markedly after HS and did not decrease significantly within the first 6 h of recovery, but showed a significant reduction after 12 h of recovery. Notably, the results showed that the enrichment of ubiquitinated insoluble proteins was significantly higher in *ubp1abc* mutants than in the WT at 12 h following HS recovery (**new Fig 6f and Supplementary Fig 4**), which correlates with the reduced autophagy activity in *ubp1abc* mutant (**Fig 6c, 6d and Extended Fig. 12**).

Specific comments:

- In extended data Fig.4, analyses of ATG18 do show some colocalization. Why use another marker of SGs? (UBP1c for all expect for ATG18: RBP47?)

Response: Thank you for your suggestion. We have revisited the colocalization analysis of ATG18 with SGs using the UBP1c-mCherry marker (**Extended Data Fig. 2b**).

- Fig. 2b-d, please define CK

Response: We have replaced "CK" with "control" throughout the manuscript.

- Fig. 2g, please provide quantification of at least 3 independent experiments

Response: We have conducted quantitative analysis of the levels of ATG and UBP1 proteins in the supernatant and precipitate on different biological replicates and have now labeled the relative intensities in the corresponding figures.

- Fig.3d,e: why is the disappearance of UBP1c SGs is much more rapid in atg5-1 than in WT in recovery experiments? Please provide quantification and discussion of this result.

Response: Thanks for your comments. We are very sorry for our mistake. There was an error in the labeling of images in the *atg5-1* background, where it was incorrectly indicated as a '3-hour recovery' when it should have been '6-hour recovery'. This mistake has been corrected in the revised manuscript. In addition, we have provided detailed statistical results on the number of SG in WT and *atg5-1* mutants (**new Fig. 3e**).

- Fig.4c, left panel: the authors mention finding ATG8f on membraneless structure. From the data provided I disagree: ATG8f particles seem to decorate a cup-shape structure resembling the initial autophagy compartment (isolation membrane, phagophore). The authors need to provide additional images. These will provide important information on the nature of the ATG8f puncta that massively accumulate as soon as after 1h of recovery.

Response: We have carefully considered your question and examined more immuno-TEM images. We found that the gold particles were predominantly found on electron-dense condensates at 3 h post HS recovery, with occasional detection on cup-shaped phagophores, suggesting the initiation of autophagosome formation at this stage (**new Extended Fig. 10**). Together with

the confocal observation showing the absence autophagic bodies inside the vacuoles at 3 h (**Fig. 4d**), these results suggested that autophagy is only in its initial stage at this time point.

Fig.4d, need quantification of 3 independent experiments. What is MS-C medium: solid plates? Liquid? What about 'untreated' plants? Were they still put in liquid medium etc? Why would autophagy be reduced in plants primed for HS? It seems to go in the opposite direction of the authors conclusion that translocation of ATG proteins to SGs facilitates the activation of autophagy.
Response: Thanks for your comments. In the original Figure 4d, we had implemented a carbon starvation treatment subsequent to heat stress to illustrate the inhibition of the autophagy pathway during the heat stress phase and the early recovery stage, with subsequent activation during the later stages of recovery. We agree with your valuable insight and that of other reviewers that introducing an additional stressor, such as carbon starvation, complicates the interpretation of the results. After careful deliberation, we have decided to remove this result (Fig. 4d in previous manuscript). Instead, we replaced this experiment with concanamycin A treatment to demonstrate the accumulation of autophagic bodies inside vacuoles after HS (**Fig. 4d**). Moreover, we have also demonstrated that the HS-primed plants exhibited an enhanced accumulation of autophagic bodies inside vacuoles compared to the untreated controls (**new Fig 5e and f**), and that the *ubp1abc* mutant exhibited reduced autophagy activity (**Fig 6c, 6d and Extended data Fig 12**).

- How is heat stress treatment performed/controlled? Are the unstressed plants also put in liquid medium in a water bath at 22C as control? What about light conditions during HS?
Response: We have added a detailed description about the heat treatment in the methods section (**Page 17-18, highlighted in magenta**).

- From Fig. 4 the authors conclude that: (line 207-208) ' These findings imply that the autophagy pathway is inhibited during the HS phase and the early stage of recovery, whereas it is activated in the later stage of HS recovery.' Results presented in that figure do not support this conclusion, in my opinion.
(1) The massive accumulation of ATG8 puncta suggest a large induction of autophagy as soon as 1h of recovery and peaking at 6 h of recovery (Fig. 4a);
(2) from the one picture shown in Fig.4c left panel, ATG8f is found on isolation

membrane/phagophore in these conditions (showing at least start of autophagosome formation); (3) HS-primed plants show reduced GFP-ATG78f degradation compared to untreated plants upon -C conditions suggesting lower autophagy flux.

Based on (1) and (2), we can suppose that autophagy is induced quite rapidly after HS recovery. This has to be experimentally tested by performing GFP-ATG8 degradation assays upon HS and after a time course of recovery in rich conditions; additionally, using concanamycin A in the same conditions as performed in Fig. 4a,b, should inform on the amount of autophagosomes formed and translocated in the vacuole upon each of these conditions/time (again, to follow flux).

Based on (3), it seems that HS-primed plants actually have lower autophagy flux than untreated plants: why would the authors then conclude that (lines 215-216): 'release of ATG proteins from disassembled SGs aids in initiating the activation of autophagy during the HS recovery phase'. I am not sure to follow the rational here. This conclusion is in clear contradiction with results that are presented in Fig.4d. The authors should properly discuss this result and explain why they tested -C induced autophagy after HS recovery? Again, the authors should assess autophagy flux using the same assay but looking at before HS, after HS and in a time course of recovery, compared to plants put in the same conditions without HS.

Response: Thank you very much for your constructive suggestion. We have tried several times to conduct GFP-ATG8 cleavage assay to quantify the changes in autophagy flux before and after HS. Unfortunately, we were not able to reach a consistent conclusion, because we found that it was hard to detect the free GFP protein bands cleaved from GFP-ATG8 during the 12 h recovery period after HS. This challenge arose when the HS-primed plants were put in the standard 1/2 MS medium with 1% sucrose for recovery. Therefore, following your suggestions, we shifted our approach to apply concanamycin A treatment to HS-primed plants, which allowed us to observe the accumulation of autophagic bodies inside vacuoles during the recovery from HS (**Fig. 4d**).

In the original Figure 4d, we had implemented a carbon starvation treatment subsequent to heat stress to illustrate the inhibition of the autophagy pathway during the heat stress phase and the early recovery stage, with subsequent activation during the later stages of recovery. We agree with your valuable insight and that of other reviewers that introducing an additional stressor, such as carbon starvation, complicates the interpretation of the results.

After careful deliberation, we have decided to remove this result (Fig. 4d in previous manuscript).

Instead, we replaced this experiment with concanamycin A treatment to demonstrate the accumulation of autophagic bodies inside vacuoles after HS (**Fig. 4d**). The obtained results demonstrated that the EYFP-ATG8f-labeled autophagic bodies inside vacuoles were barely detectable during the first 3 h of recovery (**Fig. 4d, e**), with only a few observed at 6 h post-recovery (**Fig. 4d, e**). However, a significant number of autophagic bodies inside vacuoles could be clearly observed at 9 and 12 h post-recovery (**Fig. 4d, e**). Moreover, we have also demonstrated that the HS-primed plants exhibited an enhanced accumulation of autophagic bodies inside vacuoles compared to the untreated controls (**new Fig 5e and f**), and that the *ubp1abc* mutant exhibited reduced autophagy activity (**Fig 6c, 6d and Extended data Fig 12**). Together, these findings imply a correlation between the temporal regulation of autophagosome formation and the dynamics of assembly and disassembly of ATG-containing SGs. We have revised the manuscript by providing the above new data including **Fig 4d, 4e, 5e, 5f, 6c, 6d and Extended data Fig 12,** and have also rephrased the related descriptions in the text (**Page 9-11, highlighted in magenta**).

- Fig. 5

Autophagy involved in disassembly of SGs (after HS) in mammals. No colocalization between ATG8f and SGs. What about other ATG8s? Other components of the autophagy machinery that are more ubiquitous than ATG8 were multiple isoforms could be involved in dedicated types of autophagy? ATG18 for instance.

Response: Thank you for your comments. Previous studies in mammalian cells have shown that autophagy is involved in clearance of persistent (long lived) SGs under conditions of disease mutations or prolonged stress (Buchan et, al. 2013. Cell), but does not participate in the disassembly of transiently formed SGs (Wang et, al. 2019. Mol Cell). A more recent study demonstrated that ubiquitination of G3BP1, rather than autophagy, plays a central role in the disassembly of heat shock-induced SGs (Gwon et, al. 2021. Science). Consistently, we observed no significant difference in the assembly and disassembly dynamics of SGs between wild-type and *atg5-1* mutant plants following HS treatment (**Fig 3d and 3e**). These findings support the absence of colocalization between ATG8 and SGs during HS recovery (**Fig. 5a**), as SGs

are transiently induced in this scenario.

We have examined the localization changes of other ATG8 members during heat recovery, and they appeared to exhibit similar localization patterns during the HS recovery phase (**Extended data Fig. 9a**). Given the apparent lack of involvement of autophagy in the disassembly of SGs during the recovery stage from HS, we hypothesize that these ATG8 members might not likely to colocalize with SGs during the recovery stage. The involvement of general autophagy or specific types of selective autophagy, potentially regulated by different isoforms of ATG proteins like ATG8 or ATG18, in the clearance of SGs induced by other stressors, remains to be elucidated in plants. Therefore, we prefer to leave this question open rather than draw an exclusive conclusion here, and hope the reviewer can agree with us to consider this study as a future work.

- Line 226: 'Disassembly of SGs is not autophagy dependent' (Fig.3d,e): actually the disassembly appears much faster in the atg5-1 mutant: why ?
Response: Thanks for your comments. We are very sorry for our mistake. There was an error in the labeling of images in the *atg5-1* background, where it was incorrectly indicated as a '3-hour recovery' when it should have been '6-hour recovery'. This mistake has been corrected in the revised manuscript. In addition, we have provided detailed statistical results on the number of SG in WT and *atg5-1* mutants (**new Fig. 3e**).

- Fig.5b, what is control: HS-treated plants? Untreated plants? Why is NBR1 forming many puncta in untreated conditions (or in HS conditions): is there also an accumulation of NBR1 in SGs? At 3h: many co-localizations: seem to indicate that autophagy is already induced. Why not look at concA in these conditions rather than after 12h of recovery? Fig. 5e lacks proper control (no priming) and without concA.
Response: Thanks for your comments. The control means the plants were left at 22°C water bath for 1 h. We have revised this figure and figure legends to make the label clearer. Following your suggestion, we have reperformed the experiments with proper controls with or without Conc A treatment to observe the accumulation ATG8/NBR1-labeled autophagic bodies inside vacuoles. This data is now shown in new **Fig. 5e** with appropriate description in the text (**Page 10-11, highlighted in magenta**).

In previous studies (Thirumalaikumar, et. al. 2020. Autophagy.; Lin et.al.

2020. Plant signaling behavior.), NBR1 has also been shown to exhibit punctate structures under normal conditions. It cannot be ruled out that a small amount of protein aggregates labeled by NBR1 exist in cells under normal conditions. However, since SGs are absent from the cytoplasm under normal conditions, it is unlikely that the observed NBR1 puncta correspond to SGs in this state. During heat stress phase, both SGs and NBR1 puncta co-exist in cytoplasm. We surveyed the public available proteomic data and found that the NBR1 protein was absent in the mass spectrometry data obtained from SGs in plants (Kosmacz et.al, 2018, New Phytol; Gutierrez-Beltran et al, 2021, EMBO J), which might indicate the absence of colocalization between NBR1 and SGs puncta. However, we need to admit that we still lack any experimental evidence to show the correlations between SGs and NBR1-puncta during heat stress phase, and hope the reviewer can agree with us to consider this study as a future work.

Although EYFP-ATG8f punctate showed colocalization with NBR1 at 3 h post HS recovery, we have not observed an obvious accumulation of autophagic bodies inside vacuole at this time (**Fig. 4d**). Coinciding with the absence of gold particle labeling on double membrane autophagosomes (**Fig. 4c and Extended Fig. E10**), these results suggest the initiation of autophagosome formation rather than the delivery of autophagosome to vacuoles at 3 h post HS recovery.

- Lines 244-245: 'The above results suggest that SGs may potentially safeguard ATG proteins, whose release during the HS recovery phase facilitates the rapid activation of autophagy for cell survival.' The above results or conclusions from the authors actually suggest that HS somewhat reduces autophagy (when seedlings are primed with HS, they induce less autophagy).

Response: We thank the reviewer for highlighting the discrepancy in the interpretation of the results. The original Figure 4d, which included a carbon starvation treatment following heat stress, was intended to illustrate the inhibition of the autophagy pathway during the heat stress phase and the early recovery stage, with subsequent activation during the later stages of recovery. However, we acknowledged the reviewer's comments about the complexity introduced by this additional stressor. In light of this feedback, we have removed the original Figure 4d from the manuscript. Our revised approach involves the use of concanamycin A treatment to assess autophagy activity. This treatment was applied to plants co-expressing NBR1-GFP and mCherry-ATG8f, revealing

an increased accumulation of NBR1/ATG8 bodies in the vacuoles of heat-stressed plants (**new Fig 5e and f**). Furthermore, we observed a significant reduction in the accumulation of EYFP-ATG8f-labeled autophagic bodies in the *ubp1abc* mutant compared to wild-type plants during the recovery phase post-heat stress, indicating a delay in autophagy activation in the mutant (**Fig 6c, 6d and Extended data Fig 12**). We hope the reviewer will find our revisions satisfactory and agree with our conclusion that autophagy is indeed activated during the recovery stage following heat stress, and that the dynamics of SGs play a positive role in this process.

- Fig.6a,b is not a direct measurement of autophagy. The authors need to look at flux rather than only number of ATG8 structures. There may be less structures because more autophagosomes already translocated in the vacuole. Again, the authors need to use concA to assess this.

Response: Following your suggestion, we have included the results of the Conc A treatment during recovery phase in both EYFP-ATG8f/WT and EYFP-ATG8f/*ubp1abc* mutant. The accumulation of EYFP-ATG8f-labled autophagic bodies inside vacuoles was notably reduced in the *ubp1abc* mutant than the WT plants at 9 and 12 h post-HS recovery, suggesting delayed autophagy activation in the *ubp1abc* mutant during the HS recovery phase. These new results are shown in **Fig. 6c, d** with appropriate description (**page 11, highlighted in magenta**).

- Fig.6c: the authors suggest that there is more ubiquitinated proteins in the ubp1abc mutant: this is not obvious to me, the authors need to provide a quantification of these experiments with additional independent replicates and statistical analyses. Further, the authors propose that HS induce the ubiquitinated aggregates and that autophagy is involved in their clearance, more efficiently because ATG proteins are stored in SGs in these conditions. The authors should provide data that support this hypothesis. Specifically, the authors should show how HS affects the level of ubiquitinated proteins (1h 38°C) and then follow the level of ubiquitinated proteins upon a time course of recovery +/- formation of SGs (*ubp1abc* mutant) or autophagy (atg mutant).

Response: We have examined the abundance of ubiquitinated insoluble proteins in the WT and *ubp1abc* mutants during the HS and HS recovery phases (**Fig. 6f-g**). The obtained results showed that the ubiquitinated insoluble protein increased markedly after HS and did not decrease significantly within

the first 6 h of recovery, but showed a significant reduction after 12 h of recovery. Notably, the results showed that the enrichment of ubiquitinated insoluble proteins was significantly higher in *ubp1abc* mutants than in the WT at 12 h following HS recovery. This aligns with the appearance of circular autophagosomes and the accumulation of autophagic bodies at 9-12 h post-HS recovery. These new results are shown in **Fig. 6f-g** and **Supplementary Fig S4** with appropriate description in the text (**Page 12, highlighted in magenta**).

- The authors should also provide more elements, at least in the discussion, about the potential molecular mechanisms by which these particular ATG proteins are recruited and stored in SGs. For instance, the authors show a direct interaction between UBP1a and several ATG proteins, specifically during HS. By exploring the sequences/3D structure of SGs-stored ATG proteins, could the authors identify a specific domain that binds to UBP1a? How is this interaction regulated upon HS? In the absence of either of the 3 UBP1 proteins, the association of ATG proteins with SGs is significantly reduced but about a third of the puncta are still formed: this implies that UPB1 is not solely responsible for ATG interactions with SGs. How many SGs are still formed in the ubp1abc triple mutant in the same conditions? The authors provide part of the answer in Fig.S1, by looking at RBP47B in protoplasts of the ubp1abc mutant after 30 min of HS. Unfortunately, the image shown in Fig.S1 could be of better quality, further, for consistency and proper conclusion, HS should be performed after 1h in roots of Arabidopsis.

Response: Thank you very much for your valuable comments. Following your suggestion, we have now obtained transgenic plants stably expressing mCherry-RBP47b in WT and *ubp1abc* mutant, and have confirmed the reduction of HS-induced mCherry-Rbp47b foci in the roots of *ubp1abc* mutant compared to that in WT seedlings. The new results are shown in **Supplementary Fig. 3 e-g**.

Furthermore, we have discussed the potential mechanisms of ATGs recruitment to SGs (**page 12-13, highlighted in magenta**). A scaffold-client working model has been employed to elucidate the role of core components (scaffold proteins essential for SGs formation) and client components (biomolecules recruited via scaffold-client interactions) in SGs formation as described in previous studies. In this model, SG assembly is driven by oligomerization and phase-separation of scaffold proteins to from a scaffold-

scaffold-RNA bond network. Subsequently, client proteins are recruited to scaffold-scaffold binding sites by interaction with scaffold. In this study, we demonstrated that ATG proteins might not contribute to the driving force of SG assembly (**Fig. 3d and 3e**), suggesting that they act as client proteins within SGs. Given that the *ubp1abc* triple mutant showed an obvious defect in the formation of HS-induced SGs, as indicated by the core component mCherry-Rbp47b (**Supplementary Fig. S3e**), the reduction in ATGs-labeled puncta in *ubp1abc* mutant might be attributed to the defect in SGs assembly. Therefore, despite observing an interaction between ATG proteins and components of SGs under HS conditions *in vivo* (**Fig. 2**), the precise scaffold components that are directly responsible for recruiting ATG proteins have yet to be identified.

- The authors discuss about the different environmental signals leading to different populations of SGs. Similarly, different stresses/signals cause different types of autophagy, notably though the recruitment of specific cargo and the use of specific ATG8 proteins. In this paper, the authors solely based their autophagy measurement on ATG8f. Is there a particular reason for that? What about other ATG8s? Are they stored in SGs? Are they involved in activating autophagy upon HS stress or HS stress release?
Response: Thank you for your comments. We have also examined the localization changes of other ATG8 members during heat recovery, and they appeared to exhibit similar localization patterns during the HS recovery phase (**Extended data Fig. 9a**). Given the apparent lack of involvement of autophagy in the disassembly of SGs during the recovery stage from HS (**Fig. 3d**), we hypothesize that these ATG8 members might not likely to colocalize with SGs during the recovery stage. The involvement of general autophagy or specific types of selective autophagy, potentially regulated by different isoforms of ATG proteins like various ATG8 members, in the clearance of SGs induced by other stressors, remains to be elucidated in plants. Therefore, we believe it is prudent to leave the question of the specific functions of ATG8 isoforms in heat stress as an open question, and hope the reviewer can agree with us to consider this study as a future work.

Previous analyses showed that autophagy is activated upon HS, how does that compare to the mechanism/process that the authors are presenting here? If ATG proteins are stored in SGs how can they participate in autophagy induction during HS? Are ATG proteins stored only transiently in these SGs? Why?

Response: Previous studies on heat-activated autophagy have primarily focused on two aspects: transcriptional regulation and cellular activity. At the transcriptional level, it has been demonstrated that autophagy genes are significantly upregulated following heat shock (Zhou et al. 2014. Front Plant Sci; Sedaghatmehr et al. 2019. Plant Cell Environ), which enhances autophagy levels and aids in cell survival. At the cellular level, autophagy is inhibited rather than activated during the heat shock phase (Jung et, al. 2019. J Exp Bot). After the heat shock is alleviated, there is a prolonged activation of autophagy (Thirumalaikumar, et, al. 2019, Autophagy). This is consistent with our conclusion. In this manuscript, we found that autophagosomes were absent in cytoplasm and vacuole during heat shock phase (**Fig 4a and Extended Data Fig. 9b**), and ATG8-PE level was down-regulated during heat shock phase (**Extended Data Fig. 12**), consistent with previous reports (Jung et, al. 2019. J Exp Bot). These results together suggest that autophagy is inhibited during heat shock. However, after the heat shock disappeared, ATG8-PE levels were quickly up-regulated (**Extended Data Fig. 12**) and a significant number of autophagic bodies inside vacuoles could be clearly observed (**Fig. 4d**).

Here, we described a dynamic localization change process of ATG proteins during HS and recovery phases. Autophagy proteins are recruited to SGs during HS phase, whereas ATG proteins are returned to the cytoplasm accompanying with the disassembly of SGs during recovery phase, facilitating the activation of autophagy post HS recovery phase (**Fig. 3 and Extended data Fig. 8**). Our study sheds new light on the subcellular process of ATG protein mobilization that regulates autophagy activity in response to HS, and highlights the important roles of SGs in plant autophagy activation during HS.

Reviewer #3 (Remarks to the Author):

Li et al. demonstrate that during mild heat shock (HS) conditions, essential autophagy-related (ATG) proteins relocate to stress granules (SGs), resulting in a temporary suppression of autophagy. Following the alleviation of HS, these ATG proteins are released into the cytoplasm, aiding in the removal of ubiquitinated proteins. While the study is interesting, some of its conclusions lack sufficient supporting data. Several points are needed to be addressed to support their conclusions more firmly.

Response: We appreciate your time in carefully reviewing our manuscript and your constructive comments. We have thoroughly considered your comments and made significant revisions accordingly.

Major comments:

1-The study applies a mild heat shock (HS) treatment of 38°C for 1 hour often referred to as priming HS (also in Fig.4d). Yet, the formation of SGs and the activation of autophagy can be triggered by a wide range of HS intensities. To clarify and strengthen their findings, the authors should determine whether the observed effects are unique to mild HS conditions or if they apply broadly to different HS intensities, including severe HS scenarios cited in their other work. Should these effects be specific to mild HS, the authors are encouraged to explore or explain the biological importance and regulatory mechanisms of this specificity.

Response: Thank you very much for your constructive comments. Following your suggestion, we have conducted treatments at a variety of temperatures ranging from 34°C to 40°C on the plant co-expressing UBP1-mCherry and various ATG-GFP fusions. Given that 34°C is recognized as the threshold temperature for SG formation in Arabidopsis (Hamada et, al. 2018, J Cell Sci), we have chosen 34°C as our baseline treatment temperature. Our obtained results demonstrated that upon HS at 34°C for 1 h, while ATG protein aggregates and SGs began to appear in the cytoplasm, their co-localization was minimal. However, as the temperature rises, the co-localization of ATG proteins and SGs gradually increased, indicating a stronger association of ATG proteins and SGs within the temperature range of 38°C to 40°C (**Extended Data Fig. 2c-h**). Stimulation at higher temperatures at 42°C to 45°C for 1 h led to cell death of these seedlings, thereby precluding further investigation at higher temperature. These new results are shown in **Extended Data Fig. 2c-h**

with proper description and discussion in the revised manuscript (**page 5 and 14, highlighted in magenta**).

2-In Figure 3b/c, the authors demonstrate a significant decrease in puncta of ATG13a, ATG6, and ATG5 GFP fusions in the ubp1abc mutant compared to WT plants following 1h of HS, highlighting SG assembly's role in the translocation of ATG proteins to SGs. However, to support this conclusion, it's crucial to ensure that the observed differences aren't due to lower baseline expression or protein levels of ATGs in the mutant. The authors should present the basal expression and abundance of these ATGs in both Col-0 and the ubp1abc mutant using both qRT-PCR and western blotting.

Response: Thank you for your valuable suggestion. We have now included additional Western blotting data demonstrating that the expression levels of ATG13a, ATG6, and ATG5-GFP are comparable between the WT and the *ubp1abc* mutant. These updated results are shown in **Supplementary Fig 3h-j**.

3-Figure 3d presents representative images illustrating the gradual decrease in the number of stress granules (SGs) during recovery from heat shock (HS); however, the data lacks quantification, and this should be provided. Furthermore, although there is no difference in the formation of SGs puncta upon HS treatment, the number of SGs significantly decreased or (nearly absent) in the *atg5-1* background at 3h after recovery. This observation suggests a crucial role of ATG proteins, including ATG5, in the maintenance and persistence of SGs beyond their initial formation. Additional comments on this observation are recommended.

Response: Thanks for your suggestion. We have quantified the changes in SGs numbers during HS and recovery phase (**Fig. 3e**).

Additionally, we are very sorry for our mistake. There was an error in the labeling of images in the *atg5-1* background, where it was incorrectly indicated as a '3-hour recovery' when it should have been '6-hour recovery'. This mistake has been corrected in the revised manuscript.

4-Extended Data Fig. 1e: The authors did not use concanamycin A (as otherwise, it would have been mentioned in the legend or methods). Without ConA, the rare puncta formation is likely observed due to autophagosome degradation from high acidic vacuolar PH. To ensure the conclusion of the

absence of autophagosomes during mild HS (in both Extended Data Fig. 1e and Figure 4a), the authors should repeat the experiment by including ConA and appropriate controls.

Response: Thank you very much for your constructive suggestion. Following your suggestions, we have now conducted concanamycin A treatment to HS-primed plants, which allowed us to observe the accumulation of autophagic bodies inside vacuoles during the HS and recovery phases (**new Fig. 4d and Extended data Fig. 9b**).

The obtained results demonstrated that the EYFP-ATG8f-labeled autophagic bodies inside vacuoles were barely detectable during HS phase and the first 3 h of recovery (Fig. 4d, e), with only a few observed at 6 h post-recovery (**Fig. 4d, e**). However, a significant number of autophagic bodies inside vacuoles could be clearly observed at 9 and 12 h post-recovery (**Fig. 4d, e**). These findings imply a correlation between the temporal regulation of autophagosome formation and the dynamics of assembly and disassembly of ATG-containing SGs. These new data are provided in **Fig 4d, e and Extended data Fig. 9** with appropriate description in the text (**Page 9-10, highlighted in magenta**).

5-Figure 6a/b: the authors discuss that circular autophagosomes labeled by EYFP-ATG8f were delayed in ubp1abc mutant in comparison to WT plants However, since ConA wasn't used, it's unclear if these puncta are truly autophagosomes or any other types of condensates/aggregates. I am not aware of any reports showing such massive autophagosome accumulation without the use of ConA. Additionally, to confirm the delayed autophagic activity, further experiments beyond microscopy, such as autophagic flux assays and immunoblotting for NBR1 and ATG8 levels, are necessary.

Furthermore, the authors should examine and discuss any potential differences in autophagic activity between EGFP-ATG8f/*ubp1abc* mutant and EGFP-ATG8f under control conditions. This analysis could influence the interpretation of the results presented in Figure 6a/b.

Response: Thanks for your constructive comments. Following your suggestion, we have included the results of the Conc A treatment during recovery phase in both EYFP-ATG8f/WT and EYFP-ATG8f/*ubp1abc* mutant. The accumulation of EYFP-ATG8f-labled autophagic bodies inside vacuoles was notably reduced in the *ubp1abc* mutant than the WT plants at 9 and 12 h post-HS recovery, suggesting delayed autophagy activation in the *ubp1abc* mutant during the HS

recovery phase. The new results are depicted in **Fig. 6c, d** with appropriate description (**Page 11, highlighted in magenta**).

Moreover, we have tried several times to conduct GFP-ATG8 cleavage assay to quantify the changes in autophagy flux before and after HS. Unfortunately, we were not able to reach a consistent conclusion, because we found that it was hard to detect the free GFP protein bands cleaved from GFP-ATG8 during the 12 h recovery period after HS. This challenge arose when the HS-primed plants were put in the the standard 1/2 MS medium with 1% sucrose for recovery. Therefore, we have changed to do immunoblotting with an anti-ATG8 antibody to assess the lipidated and non-lipidated forms of ATG8. The lipidated ATG8 (ATG8-PE adduct) mainly labels the forming autophagic vesicles and completed autophagosomes, and the ratio of ATG8–PE to ATG8 is indicative of autophagy activity. The newly obtained results demonstrated that the ATG8-PE adduct appeared during the post-HS recovery period, but the ATG8-PE/ATG8 ratio was considerably lower in the *ubp1abc* mutant than in WT plants (**new Extended Data Figure 12**). Additionally, we have also detected the protein levels of NBR1 and ATG8, and found that the accumulation of NBR1 and ATG8 in *ubp1abc* was significantly higher than in WT during the HS recovery phase (**new Fig. 6e**). Together, these results suggest that disruption of SG assembly in *ubp1abc* mutant leads to a delay in autophagy activation during post-HS recovery period.

To verify if macroautophagy activity in the *ubp1abc* mutant is affected, we subjected EYFP-ATG8f and EYFP-ATG8f/ *ubp1abc* plants to carbon starvation treatment supplemented with Conc A to monitor the number of autophagosomes inside the vacuoles. The results showed no significant difference in their macroautophagy activity. The new results are shown in **Extended Data Fig. 11** with appropriate description in the text (**page 11, highlighted in magenta**).

6- Figure 4a, again, the authors did not use ConA (see the comment above).
Response: Thank you for your comment. This question has already been addressed above (please refer to question 4).

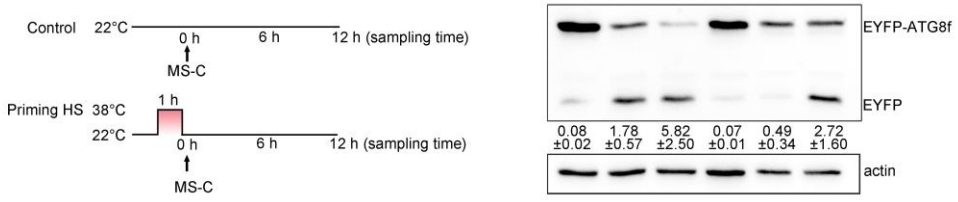
7-Figure 4d. Surprisingly, higher autophagic activity is detected under control conditions at 6 and 12 hours (EYFP/EYFP-ATG8f ratios of 1.15 and 3.59) compared to post-heat treatment (EYFP/EYFP-ATG8f ratios of 0.06 and 1.17). The authors should discuss potential reasons for this unexpected finding and

include control images for autophagosome detection, as in Figure 4a, for better clarity. While EYFP/EYFP-ATG8f effectively indicates autophagic activity, supporting this data with additional metrics is crucial, especially by assessing NBR1 and ATG8 levels through immunoblotting, for which reliable antibodies are available at Abcam.

Response: Thanks for your valuable comments. In the original Figure 4d, we had implemented a carbon starvation treatment subsequent to heat stress to illustrate the inhibition of the autophagy pathway during the heat stress phase and the early recovery stage, with subsequent activation during the later stages of recovery. We agree with your valuable insight and that of other reviewers that introducing an additional stressor, such as carbon starvation, complicates the interpretation of the results. After careful deliberation, we have decided to remove this result (original Fig. 4d in previous manuscript). Instead, we replaced this experiment with concanamycin A treatment to demonstrate the accumulation of autophagic bodies inside vacuoles after HS (**new Fig. 4d**). We also found that the HS-primed plants exhibited an enhanced accumulation of autophagic bodies inside vacuoles compared to the untreated controls (**new Fig 5e and f**).

Following your suggestion, we have also conducted immunoblotting with an anti-ATG8 antibody to assess the lipidated and non-lipidated forms of ATG8. The newly obtained results demonstrated that the ATG8-PE adduct appeared during the post-HS recovery period, but the ATG8-PE/ATG8 ratio was considerably lower in the *ubp1abc* mutant than in WT plants (**new Extended Data Figure 12**). Additionally, we have also detected the protein levels of NBR1 and ATG8, and found that the accumulation of NBR1 and ATG8 in *ubp1abc* was significantly higher than in WT during the HS recovery phase (**Fig 6e**). Together, these results suggest that autophagy activity is boosted during the post-HS recovery phase, and disruption of SG assembly in *ubp1abc* mutant leads to a delay in autophagy activation after HS.

Fig. 4 d in Previous manuscript



Original Fig 4d. The 5-day-old EYFP-ATG8f plants were first subjected to treatment at 38°C or left untreated, and then transferred to MS-C medium to induce autophagy. Samples were collected at the indicated time points followed by immunoblotting analysis. The values represent the ratio of EYFP to EYFP-ATG8.

8-In lines 238-240, the author notes the role of NBR1 in releasing autophagic activity to eliminate proteins marked by NBR1 during recovery from heat stress (HS). To further validate this claim, measuring NBR1 levels in the ubp1abc mutant during HS and recovery is essential, as they serve as an indicator of autophagic activity and its participation in this process.

Response: Thanks for your suggestions. Following your advice, we have detected the accumulation of NBR1 in WT and *ubp1abc* during HS and recovery stages and found that the accumulation of NBR1 in *ubp1abc* was significantly higher than in WT during the HS recovery phase. The new results are depicted in **Fig. 6e** with a proper description (**Page 11-12, highlighted in magenta**).

Minor comments:

1) The use of UBP1a for BiFC and Co-IP experiments (Figure 2a,b) and UBP1c for co-localization studies (Figure 1b) raises questions about the rationale behind choosing different UBP1 isoforms for these experiments. Given that UBP1a, UBP1b, and UBP1c are distinct proteins located at the core of stress granules in Arabidopsis thaliana, it would be helpful if the authors could clarify why specific isoforms were selected for particular assays.

Response: UBP1a, UBP1b, and UBP1c are members of the same homologous protein family, exhibiting high protein sequence conservation, and all can serve as markers for SGs. Initially, we randomly selected two members (UBP1a and UBP1c) to test the possible associations and colocalizations between these ATG proteins and SG components. It is important to note that, in addition to UBP1a, we have also confirmed associations between UBP1c and several ATG proteins in our BiFC assays under heat stress condition, although this data is not presented in the manuscript. These findings suggest that ATG proteins can associate with UBP1 proteins *in vivo* without apparent preference.

2) Line 287 and Fig 3a: “These ATG puncta were induced by HS, but not by sodium arsenite (Fig. 3a), indicating the specific involvement of HS-induced SGs in the regulation of autophagic activity, but not other stress-induced SGs”.

This is a very strong generalized statement regarding other abiotic stresses. Recent studies have demonstrated the induction of autophagy and SG formation by various stressors, such as carbon starvation, oxidative stress, salt, drought, and cold. It's likely that proteins such as ATG5, ATG6, and ATG13 also translocate to SGs under other stress conditions. Further experiments are needed to provide evidence for this hypothesis. I, therefore, recommend authors moderate the tone.

Response: Thanks for your valuable suggestion. We have rephrased the description to tone down this conclusion (**Page 7, highlighted in magenta**).

In addition, following your suggestion, we have subjected the transgenic plants to various abiotic stresses to induce SGs, including salt stress, drought stress (simulated by 20% PEG4000), osmotic stress. The obtained results showed that all these abiotic stresses were able to induce the formation of SGs, whereas no significant accumulation of autophagy proteins on SGs puncta was observed. The new results are depicted in **Extended Data Fig. 7b** with appropriate discussion in the revised manuscript (**Page 14, highlighted in magenta**).

3) In Figure 1c, "n=10" needs clarification: does it refer to 10 roots, 10 cells, or something else? Additionally, the analysis should specify the size of the root area studied for co-localization

Response: we have now clarified this detailed information in the figure legends of the revised manuscript.

4) Co-localization data shown in Figure 2a, Extended data figures 2a,b and 3a,b should be quantified.

Response: We have introduced co-localization analysis on these images by calculating their Pearson correlation coefficients. The results are presented in the corresponding images.

5) Extended data Fig. 1b- Please include the control images for roots without heat stress.

Response: We have now added the confocal images of full roots in control conditions (**Extended Data Fig 1 b-d**).

6) Line 140-141- The reduction in the levels of ATG proteins and UBP1a-FLAG in the supernatant should be quantified.

Response: We have analyzed the relative grayscale intensities for the ATG and UBP1 proteins in the supernatant and precipitate on different biological replicates of **Fig. 2h** and **Extended Data Fig 6**.

7) Figure 6C- It is important that authors provide quantitative analyses to better present/clarify the differences in the abundance of ubiquitinated proteins between ubp1abc mutants and wild-type (WT).
Response: We have repeated this experiment and have conducted quantification of the relative abundance of ubiquitinated proteins in the *ubp1abc* and WT plants. These new results are shown in **Fig. 6f-g** and **Supplementary Fig S4** with appropriate description in the manuscript (**Page 12, highlighted in magenta**).

8) Lines 154-158—Heat stress is often accompanied by oxidative stress. I recommend to discuss why arsenite treatment, which causes oxidative stress, cannot change the localization of ATG proteins.
Response: We have expanded the discussion on your points (**Page 14, highlighted in magenta**).

9) Line 224- colocation should be colocalization.
Response: We have corrected this mistake.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We appreciate your time in carefully reviewing our manuscript and your constructive feedback. We have thoroughly considered your comments and made significant revisions accordingly.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all of the points sufficiently. Congrats to this very nice work.

Reviewer #2 (Remarks to the Author):

The authors have properly addressed my initial concerns with appropriate additional experiments/results and discussion. This is a very interesting study and an excellent work which opens discussion and leads for future research.

I only have a few minor comments:

Lines 36-37: 'The autophagy pathway is responsible for eliminating the damaged components'.

Pathways other than autophagy may be involved in eliminating damaged components and autophagy is likely involved in degrading only specific proteins/damaged organelles in these conditions. Please be more sensible in your statement.

Line 170: none of the stress used triggered an accumulation of ATG puncta, even though these conditions are known to induce autophagy and thus autophagosome formation. Can the authors comment on that, did they look at GFP-ATG8 using the same settings?

Line 188: in the triple ubp1 mutant: the data show less puncta of the ATG proteins but similar protein level measured by western blot. If the presence of these proteins in SGs served safeguarding purposes they should be more thermal tolerant and proteins levels should be decreased compared to untreated conditions or to WT cells. This could be used as an element of discussion regarding lines 383-386 'further investigation is needed to ascertain whether SGs serve as temporary reservoirs, similar to a repository of mRNA, thereby safeguarding these ATG proteins from thermal denaturation and ensuring their rapid availability after the stress is alleviated'. Is the presence of ATG proteins in SGs is not for safeguarding, could the authors propose another potential function?

Typos: 'labled' instead of 'labeled', lines 212, 248, 252, 255, 262 and in Figure 5, line 872

‘Recocery’ instead of ‘recovery’, extended Fig. 12.

Reviewer #3 (Remarks to the Author):

The authors have effectively addressed my previous comments, and I am generally satisfied with the revisions made to the manuscript. However, one issue and a minor editing point remain that need further attention.

1) It appears that actin levels vary significantly across samples, with notably low expression observed in the ubp1abc-recovery 6-9h samples. Consequently, the ATG8-PE bands in these samples are very weak, which, in my opinion, raises questions about the accuracy of their quantification.

2) Extended Data Figure 12: Correct "recovery" to "recovery" (in the Figure)

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Responses to reviewers' comments

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Lines 36-37: 'The autophagy pathway is responsible for eliminating the damaged components'.Pathways other than autophagy may be involved in eliminating damaged components and autophagy is likely involved in degrading only specific proteins/damaged organelles in these conditions. Please be more sensible in your statement.

Response: Thank you for your careful review. We have rephrased the description (**page 3, highlighted in magenta**).

Line 170: none of the stress used triggered an accumulation of ATG puncta, even though these conditions are known to induce autophagy and thus autophagosome formation. Can the authors comment on that, did they look at GFP-ATG8 using the same settings?

Response: We appreciate your insightful comments. It is true that, contrary to expectations, stress conditions such as osmotic stress, salt stress, drought stress, and oxidative stress did not result in the accumulation of ATG proteins in stress granules (SGs), despite these conditions being known to induce autophagy and autophagosome formation as demonstrated in previous literatures. These observations from our and other studies suggest that heat shock-induced SGs may have a unique role in modulating autophagic activity compared to the SGs formed under other stress conditions. It also implies that different stress types might regulate autophagy via distinct pathways. We have included a discussion on this point in the manuscript (**page 16, highlighted in magenta**).

Line 188: in the triple *ubp1* mutant: the data show less puncta of the ATG proteins but similar protein level measured by western blot. If the presence of these proteins in SGs served safeguarding purposes they should be more thermal tolerant and proteins levels should be decreased compared to untreated conditions or to WT cells. This could be used as an element of discussion regarding lines 383-386 'further investigation is needed to

ascertain whether SGs serve as temporary reservoirs, similar to a repository of mRNA, thereby safeguarding these ATG proteins from thermal denaturation and ensuring their rapid availability after the stress is alleviated'. Is the presence of ATG proteins in SGs is not for safeguarding, could the authors propose another potential function?

Response: Thank you for your constructive comments. Indeed, under normal conditions, there is not much difference in the levels of ATG proteins between the WT and the *ubp1abc* mutant. However, we have yet to examine the changes in these ATG proteins following heat stress. Whether SGs protect autophagy proteins still requires further evidence, which we hope to address in our future work. We have expanded our discussion to include these considerations (**page 17, highlighted in magenta**).

Typos: 'labled' instead of 'labeled', lines 212, 248, 252, 255, 262 and in Figure 5, line 872 'Recocery' instead of 'recovery', extended Fig. 12.

Response: Thank you for your careful review. We have made corrections.

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Response: We appreciate your attention to detail regarding the variation in actin levels and its potential impact on the quantification of ATG8-PE bands. It is important to clarify that in our experiments, autophagy activity is represented by the ratio of ATG8-PE to ATG8, rather than in comparison to ACTIN. In the immunoblotting assay, an increase in total protein content would result in higher absolute levels of both ATG8 and ATG8-PE in the same sample. However, the ratio between ATG8-PE and ATG8 remains consistent irrespective of the amount of protein loadings, which is a critical point for assessing autophagy activity. Furthermore, since ATG8-PE and ATG8 bands are detected on the same membrane under identical exposure times and contrast settings, the ratio of ATG8-PE to ATG8 is a more reliable indicator of autophagy activity than comparing absolute levels of ATG8-PE to actin. This methodological approach ensures that our quantification is accurate and not confounded by variations in total protein content across samples. We hope this explanation address your concerns.

2) Extended Data Figure 12: Correct "recovery" to "recovery" (in the Figure)

Response: Thank you for your careful review. We have made corrections.

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