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To add value to publications and provide information on the review process, The Plant Cell will publish Peer Review Reports, subject to author approval, for all articles by January 2017. Reviewer anonymity will be strictly maintained. The reports which will include the most substantive parts of decision letters, anonymous reviewer comments, and author responses; minor comments for revision and miscellaneous correspondence will not be published. The text of reviewer comments and author responses will be unedited except to correct typos and minor grammatical errors (where noticed and easily corrected), and to remove minor comments. This report is published as part of a pilot program, including a small set of articles, with the approval of all respective authors and reviewers, to introduce readers and authors to the concept and test the format.

GOLGI TRANSPORT 1B (GOT1B) Regulates Protein Export from Endoplasmic Reticulum in Rice Endosperm Cells

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TPC2016-00717-RAR1	1st Revision received:	October 17, 2016
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00312-RA 1st Editorial decision – *declined* **June 5, 2016**

We have had input from multiple scientists who have expertise in membrane trafficking in plants and yeast, and have solicited post-review comments as well. All the reviewers, including the editors, share the opinion that the identification of GPA4 as an otholog of yeast Got1p required for proglutelin trafficking from the ER is very interesting and provides unique opportunities for discovering how Got1p function in COPII vesicle transport. Nevertheless, significant concerns (see summary of specific issues and reviewers' comments below) were raised regarding the interpretation and conclusions of the data presented regarding the role of GPA4/Got1p in regulating the assembly of COPII. Our present policy is to offer streamlined decisions and to not advise on the direction of the work by requesting extensive modifications or substantial additional experiments. Therefore, we do hope that the comments below are helpful to you as you continue your work or in preparing a revision for another journal.

[Specific issues and reviewer comments are listed below with author responses]

[The previous submission] was reviewed by two experts and both of them found this work to be very interesting and fit well with the scope of The Plant Cell. However, both reviewers raised some critical concerns about some of the biochemical data we presented about GPA4-OsSec23 protein-protein interaction, mainly because of the lack of proper positive and negative controls in some of the data we reported. Thus, the reviewers have suggested additional experiments to validate our observations. We evaluated the reviewers' comments and critiques very carefully and conducted a series of additional experiments in the last three months following the editor and two reviewers' suggestions. We hope that our revised MS will fully address the questions raised by the editor and the two reviewers.

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

Editor's specific issues:

Point 1. As discussed by reviewers specificity controls are required for the co-immunoprecipitation experiments in order to show that membrane solubilization is efficient and that the protein interactions observed are not the result of protein aggregation. In addition, given the potential for interaction artifacts (i.e. false positives) the BiFC assays require specificity controls. For a discussion of potential issues with the use of BiFC and for recommendations on best practices for BiFC analyses especially for membrane protein interaction studies please see http://www.plantcell.org/content/early/2016/04/20/tpc.16.00043.full.pdf Furthermore, all methods used in the manuscript need to be fully described.

> **RESPONSE: Thanks for your good suggestions. In our revised manuscript, we re-conducted the Co-IP and BiFC experiments using specificity controls as suggested. Initially, we used OsSec23a and OsSec23b as negative controls in the BiFC assay. Interestingly, we found that GPA4 interact with all three OsSec23 isoforms in this assay, although the traditional yeast two-hybrid assay only detected the interaction between GPA4 and OsSec23c. We further confirmed the interaction between GPA4 and OsSec23b /OsSec23c using the split-ubiquitin based Y2H assay. Thus, we used the Golgi-localized COG3 and COG8 proteins (a pair of interacting proteins, Tan et al., 2016, 2016, PLoS Genet. 12(7): e1006140. doi:10.1371/journal.pgen.1006140) as the negative controls. In the Co-IP assay, we used samples prepared with pre-immune IgG, or samples prepared without primary antibody as the negative controls. All methods have been fully described in our revised manuscript.**

Point 2. The observed Y2H interaction of (full-length??) GPA4 and OsSec23 is interesting but puzzling given that GPA4 is an ER integral membrane protein and the Y2H system reports the interaction of proteins within the nucleus. Additional experiments are thus required to explain how an ER membrane protein can interact with OsSec23a in the Y2H system. As suggested, use of mutant versions of GPA4 that fail to show ERES localization would also provide a specificity control for Y2H GPA4/Sec23 interaction analysis. Additionally controls for self-activation of the Y2H reporters by OcSec23a (i.e. in the absence of GPA4) are required.

> **RESPONSE: Full length GPA4 was found to interact with OsSec23c in the traditional Y2H assay, despite that GPA4 is an ER membrane protein. Similar observations have been reported for other membrane proteins, such as the interaction between Arabidopsis Maigo2 and the AtSec20 or AtUfeI (Li et al., 2006, Plant Cell 18: 3535–3547). To address the concern, we further used the split-ubiquitin based Y2H system, BiFC, and Co-IP assays to show the interaction between GPA4 and OsSec23 isoforms in our revised manuscript. The self-activation assay of OsSec23 isoforms is provided in supplemental Figure 7A.**

Point 3. As suggested by the reviewers, the quantitation of the fractionation data presented in figure 8A-D should be reanalyzed. In addition, the conclusions should be more cautiously interpreted given the potential for changes in ER membrane and protein abundance in the gpa4 mutant vs wild-type.

> **RESPONSE: Since Figure 8A and Figure 8C show similar results, thus we deleted Figure 8A and 8B in the revised manuscript. As suggested, we re-analyzed the data of the original Figure 8C (new Figure 8A) and calculated the distribution of each four fraction. The data is listed in supplemental Table 3. Our results showed that the membrane binding activity did not change significantly, but the distribution of OsSar1b and OsSar1c in each fraction changed significantly. The altered distribution of OsSar1 proteins suggests a possible defect in COPII vesicle formation in gpa4 mutant.**

Point 4. The data presented in Figure 8F that shows that OsSar1b co-immunoprecipitates with proglutelin is not sufficient evidence to make the conclusion that proglutelins are directly associated (i.e. cargos) with the COPII complex. As discussed in the reviews, additional controls for these experiments are necessary to demonstrate the specificity of the observed interaction. Immunoblot analysis of the Co-IP samples using ER membrane and lumenal proteins (for example Sec12 and PDI using available anti-Sec12 or PDI antibodies, respectively) could be used to demonstrate the specificity of the observed interaction between Sar1 (COPII) and proglutelins.

RESPONSE: To rule out the possibility of fragmented ER contamination or trapping of proglutelin in the detergent derived micelle, we included controls with only protein A agarose beads and rabbit IgG proteins in the co-IP experiments. Although there is slight contamination of proglutelin in the control experiments, much higher amounts of proglutelins were immunoprecipitated by the anti-Sar1b antibodies. Moreover, neither OsSec12b nor OsPDIL1-1 was detected in the IP samples precipitated by anti-OsSar1b antibodies. Thus, we concluded that the immunoprecipitated complexes by anti-Sar1b antibodies contain proglutelins, suggesting that proglutelins are the preloaded cargos of the COPII vesicles.

Point 5. Quantitation of the time-lapse image analysis of mCherry-GPA4 recruitment to the ERES relative to OsSar1b-GFP should be presented and how this observation relates to the function of GPA4 in COPII vesicle formation should be discussed.

RESPONSE: As hinted by the reviewer, the result of the time-lapse image analysis by itself cannot provide us sufficient information regarding how GPA4 regulates COPII vesicle formation. Thus, we decided to delete this result in our revised manuscript. This should not affect the central message of this work. Further more detailed studies are required to fully address this question.

Point 6. Lastly as discussed by the reviewers, loss of GPA4 function in the gpa4 mutant could indirectly result in a reduction in the levels of membrane associated Sec23 (Figure 8E). Thus, other experiments such as those suggested by the reviewers are necessary to convincingly conclude that GPA4 functions directly in the formation of COPII vesicles.

RESPONSE: Previous study in yeast suggested that Got1p participates in the budding step of COPII vesicles. GPA4 is a rice ortholog of yeast Got1p. We have provided the evidence that the interaction between Got1 and Sec23 proteins is conserved in eukaryotes. Further, we found that yeast Got1p could rescue the mutant phenotype of *gpa4-2*. Thus, GPA4 likely plays a conserved role in mediating the budding process of COPII vesicles in plants as well. According to the well-established model, once budding, Sar1-GTP will be hydrolyzed by Sec23 and released to the cytosol. Thus the interaction between Sar1 and Sec23 only occurs before COPII vesicle budding. In our study, we showed that GPA4, OsSar1b, and OsSec23c present in the same complex(es). Their coexistence suggests that GPA4 plays a role before the budding step. Further, GPA4 is an ERES-localized membrane protein, which directly interacts with OsSec23. This result suggests that GPA4 might function cooperatively with OsSar1b in facilitating the recruitment of OsSec23c to form the pre-budding complexes of COPII vesicles. Consistently, less amount of OsSec23c was immunoprecipitated by anti-Sar1b antibodies. Based on these results we propose that GPA4 likely function to facilitate the assembly of COPII vesicles.

This suggested experiment to reconstitute COPII vesicles in vitro would require functional recombinant Sar1, Sec23-Sec24, and Sec31-Sec13 proteins in the presence of microsomes. Unlike yeast, each minimal component of COPII coat generally has several isoforms in plants. To our knowledge, such attempts have not been successful in any plant systems.

Reviewer #1:

In this work, Wan and many co-workers characterized a rice mutant that accumulate precursor to glutelin, a rice storage protein deposited into vacuolar type protein body. The mutant showed the accumulation of a novel structure made with the glutelin precursor in the ER. Characterization of the corresponding gene (termed gpa4) indicated that the gene encodes a protein that is a homolog to yeast Got1p. Then biochemical and microscopic analyses of GPA4 protein and proteins required for the ER export were carried out. Based on the findings by the authors they discussed that this protein regulates protein export from the endoplasmic reticulum (ER) by facilitating the assembly of COPII vesicles.

I think the findings described in this manuscript are quite interesting and the focus of this work is quite general in plant cell biology. Thus I think the focus of this work is clearly fit to that of the Plant Cell.

However, many biochemical data presented in the manuscript are incomplete or lack appropriate controls and some data processing is inappropriate. Thus more solid biochemical analyses as well as an appropriate data processing will be necessary. In addition, critical data is missing to reach the conclusion that GPA4 protein regulates protein export from the ER by facilitating the assembly of COPII coat. The followings are the weak points of this manuscript.

Point 1. Lack of information about the characterization of antibodies used in this study. Many antibodies are generated using recombinant proteins and used in this study. However, there is no data in supplemental information that the antibodies used in this work are specific. As almost all of the antibodies used in this work were not characterized previously, data for specificity of the antibodies should be presented as supplemental data. A typical way to show the specificity of antibody is to show the result that preimmune serum will not give corresponding bands. Especially in the case of anti-OsSec23c protein, immunoprecipitated samples gave a different migration position. Although authors discussed that such different migration can be the result of phosphorylation, one cannot rule out a possibility that such different migration bands are the result of nonspecific binding or cross reaction of antibody against non-OsSec23c proteins that are present in IP fractions.

RESPONSE: Demonstration of the antibody specificity is illustrated in Supplemental Figure 7. All the newly raised antibodies used in this study detected only one major band in the western blot analyses. Meanwhile, the pre-immune serum of all the antibodies gave no corresponding band. In our revised MS, proper controls (such as samples prepared with pre-immune IgG, or samples prepared without primary antibody) were adopted, which ruled out the possibility of non-specific binding.

Regarding the nature of the larger band of OsSec23c, we noted that this band can be immunoprecipitated by both anti-GPA4 and anti-Sar1b antibodies (Figure 7C and 7D). Further, this band can be detected with both anti-OsSec23c antibodies and anti-phosphorylation antibodies (Figure 7E). These results, together with the demonstrated specificity of our antibodies, support the identity of this band as phosphorylated OsSec23c protein.

Point 2. Co-immunoprecipitation experiments lack appropriate controls. As GPA4 is a membrane protein, inclusion of detergent is essential to carry out immunoprecipitation. Combination of particular membrane protein and detergent can sometimes generate protein aggregate that can be recovered with protein A sepharose during centrifugation. Such artifact should be neglected by including proper controls in experiments. Unfortunately, such controls are not included in all the co-immunoprecipitation data presented in this manuscript. Thus all the co IP data (Figures 7C-E and 8E) in this work does not tell specific interaction as authors are discussing quite small amount (less than 1% of total) in the precipitated fraction. Therefore in all of these experiments, proper controls, such as samples prepared with pre-immune IgG, and prepared without primary antibody should be included.

RESPONSE: We have re-performed all the co-immunoprecipitation experiments. We simultaneously prepared samples with pre-immune IgG, and samples without primary antibodies. The new results support our original conclusions.

Point 3. Co-immunoprecipitation conditions were not described in the manuscript. In this work only about or less than 1% of the total corresponding proteins were used as loading controls (Figures 7C-E and 8E). As small amount of contamination may be possible when the combination of detergent and protein is inappropriate, detailed immunoprecipitation conditions should be described.

RESPONSE: Specificity controls were used in our repeated experiments to rule out the possibility of contamination. We have provided the detailed immunoprecipitation conditions in the materials and methods section.

Point 4. Lack of an appropriate control for BiFC assay. Local concentration may give false positive by BiFC assay. Thus an appropriate control is necessary in Figure 7B to provide evidence that the interaction is specific. A possible proper control is to use either OsSec23a or b because these proteins will also be targeted to the ER exit site.

RESPONSE: Using the traditional yeast two-hybrid assay, we detected the interaction between GPA4 and OsSec23c, but not OsSec23a or OsSec23b. However, we found that GPA4 interacts with both OsSec23b and OsSec23c in the split ubiquitin based Y2H assay. The interaction between GPA4 and OsSec23a cannot be determined because of the self-activation activity of pBT3-N-GPA4 (Supplementary Figure 8B). Further we found that GPA4 interacts with all

three OsSec23 isoforms in the BiFC assay. The Golgi-localized COG3 and COG8 proteins (a pair of interacting proteins, Tan et al., 2016, PLoS Genet. 12(7): e1006140. doi:10.1371/journal.pgen.1006140) were used as the negative controls. Our result showed that GPA4 does not interact with COG3, neither do three OsSec23 isoforms interact with COG8 in the BiFC assay. Further, in the Co-IP assay, we adopted proper control samples prepared with pre-immune IgG, or samples prepared without primary antibodies. From above results, we can conclude that GPA4 specifically interacts with OsSec23.

Point 5. Y2H, Co-IP and BiFC and are not sufficient to confirm specific physical interaction between GPA4 and OsSec23c. Y2H assay suggests the possibility. A positive signal from Co-IP experiments shown in Figure 7C may indicate a possible interaction. However, as described above, this data is of low quality because of the lack of proper controls and cannot be used as evidence. The problem of BiFC data is also described above.

Thus I recommend that including the data of chemical cross-linking assay to strengthen the data to support the direct interaction. In brief, the 12 DAF endosperm will be treated with membrane permeable and cleavable cross linker (such as DSP, for example). Proteins in the cross linker-treated endosperm will be denatured using SDS and then immunoprecipitation be carried out using anti-GPA4 antibody (and with control antibody for negative control). Thereafter, proteins in the precipitated samples will be treated with a chemical to cleave the cross linker (such as DTT if DSP is used) to cleave the cross linker, separated by SDS-PAGE and carry out immunoblot to detect OsSec23c and other appropriate control proteins.

RESPONSE: See our reply to the above point. For the suggested cross-linking experiment, we appreciate the reviewer's kind suggestion. Unlike yeast and mammalian cells, which is membrane permeable to DSP, rice endosperm to some extent is too thick to be penetrated. DSP might be accessible to the aleurone and subaleurone cells, while most of the inner cells of developing endosperm cannot be penetrated by DSP. Thus, it is technically very challenging and we have not seen successful application of this approach in plant systems so far, to our best knowledge.

Point 6. Using the ratio of two fractions is not a proper evaluation method to discuss the subcellular localization. In Figure 8D, the ratio of the intensity of P13 and P100 were compared in wild-type and mutants to discuss that more Sar1 proteins were recovered in P13 fraction in mutant. However the high value of P100 and P13 ratio could be observed in the following three case : Case1, intensity of the P13 is decreased while the intensity of P100 is constant; case 2, intensity of the P13 is constant while the intensity of P100 is increased; case 3, combination of both these cases.

Using the Figure 8B image, I quantified the intensities of both OsSar1b and OsSat1c in P2, P13, P100 and S100 lanes and calculated the ratio of P100 to P13 as well as the percentage of distribution in these 4 fractions. The ratio of P100 to P13 in OsSar1b was 1.7 and 1.0 in wild-type and mutant, respectively. This value well fit to the length of bar in Figure 8D. In this case about 22 and 26 % of OsSar1b seems recovered in P13 fractions from wild-type and mutant, respectively, and about 38 and 27 % in P100 fraction from wild-type and mutant, respectively. Thus in the case of OsSar1b, it may be possible that the recovery level in P13 are not different significantly in both wild-type and mutants, and different level in P100 fraction gave different values when ratio of the intensity was calculated. In the case of OsSar1c, the result of my calculation was opposite. Similar level in P100 fractions and lower level in P13 fraction from wild-type.

Thus the distribution of each four fraction after quantification of band intensities should be compared between wild-type and mutants to discuss the role of GPA4 on the recruitment of COPII coat proteins to membrane fractions.

RESPONSE: See our reply to editor's comment point 3.

Point 7. Without proper controls, Co-IP experiments using anti-OsSar1b and detection of proglutelin (Figure 8F) are meaningless.

Firstly, Sar1 is a peripheral membrane protein associated to the ER. Although Sar1 can be concentrated to ER exit site, significant proportion of Sar1 protein can be associated to non exit site ER (e.g. Figure 8G, Merge, green fluorescence). Based on the distribution of mRNA reported previously (Li et al., Cell 72, 869, 1993), proglutelin should be present not only at the luminal side of the ER exit site but also in other luminal part of the cisternal ER.

Therefore the detection of proglutelin in the IP sample using anti-Sar1 is not direct evidence that proglutelin is a cargo of COPII vesicles.

Secondly, as Sar1 is a peripheral membrane protein localized to the cytosolic face of the ER and as proglutelin is a luminal protein in the endomembrane system, the detection of proglutelin in the IP fraction using anti-Sar1 can be the result of either of the following situations. Since there is no information about the IP conditions, I assumed the following two situations. If the IP is done without detergent, immunoprecipitated fraction might contain both uncoated and partially uncoated COPII vesicles as well as fragmented and vesiclulated ER, which is generated during the homogenization of tissues. Thus it is essential to provide evidence that the IP fraction dose not contain fragmented ER. If the IP is carried out with detergent, the IP sample may contain Sar1 associated with a cargo receptor for proglutelin that bind proglutelin. However, without control experiment, one cannot rule out the possibility that a small fraction of proglutelin could not be washed out completely during immunoprecipitation, small amount of aggregate containing proglutelin is generated during the IP reaction and such aggregate was recovered in the pellet of IP reaction, or proglutelin is trapped in detergent derived micelle or vesicles which also contain Sar1 protein. Thus control data to neglect these possibilities should be provided.

RESPONSE: We performed the Co-IP assays with 0.2% NP-40 detergent. Neither OsSec12b nor OsPDIL1-1 was detected in the IP samples by anti-OsSar1b antibodies. Meanwhile, to rule out the possibility of fragmented ER contamination or the trapping of proglutelin in the detergent derived micelle, control experiments with only protein A agarose beads and rabbit IgG proteins were used. Although there was slight contamination of proglutelin in the control experiments, much higher amount of proglutelins was immunoprecipitated by anti-Sar1b antibodies as shown in Figure 8E. Thus, the immunoprecipitated complexes by anti-Sar1b antibodies contain proglutelins, suggesting that proglutelins are the preloaded cargos of the COPII vesicles.

Point 8. Lack the evidence that the gpa4 mutant defect is the assembly of COPII vesicles.

It was reported that yeast Got1p is mainly involved in the fusion of uncoated COPII vesicle to the Golgi. In this case absence of Got1p reduces the rate of the fusion of uncoated COPII vesicles to the Golgi, then reduces the level of recycled COPII budding machineries to the ER and eventually inhibits the budding of COPII vesicle from the ER.

As GPA4 is a rice ortholog of yeast Got1p, the same scenario is likely. However, authors did not favor this possibility and discuss that GPA4 is a facilitator for COPII vesicle assembly (see title). Unfortunately the data presented in this manuscript cannot distinguish these two possibilities. Thus it will be essential to include data that is conceptually similar to Figure 2 in Campbell and Schekman, PNAS 94, 837-842, 1997 using wild-type and gpa4 mutant to discuss that the major role of GPA4 protein is a facilitator of COPII vesicle assembly.

RESPONSE: According to the study of Conchon et al. (1999), Got1p is mainly involved in the fusion of uncoated COPII vesicles to the Golgi. However, in a later study (Lorente-Rodríguez et al., 2009), the got1p strain from the Research Genetics strain collection revealed that membranes prepared from this strain did not display detectable defects in any specific transport stages or in overall ER-Golgi transport. Upon re-analysis of the original got1p strain (Conchon et al., 1999), they did observe phenotypic consequences; however, these defects could not be restored by reintroduction of the GOT1 gene. These results suggest that the original got1p strain contains additional mutation(s) or a genetic background that contributes to the observed trafficking defects. Therefore, the conclusion that Got1p is involved in the fusion step needs to be further verified. Instead, Lorente-Rodríguez and its coworkers (1999) preferred the notion that Got1p participates in the budding step, rather than fusion of uncoated COPII vesicles with Golgi. Thus, these results from yeast are consistent with our proposed model.

<u>Reviewer #2:</u>

In this manuscript Wang et al characterize gpa4, a newly identified rice mutant that accumulates glutelin precursors. GPA4 encodes a protein that shares overall homology to yeast and vertebrate Got1, a tetraspanning membrane protein that functions in ER-Golgi trafficking. The authors document that loss of function gpa4 alleles cause a block in ER export of secretory proteins, alter ER structure and produce a corresponding induction of unfolded protein response stress pathways. GPA4 protein co-localizes with plant ERES and early Golgi markers supporting a function in this transport step. A new result of the study shows that GPA4 interacts with the COPII subunit Sec23 in two-hybrid and co-IP experiments. Based on these observations the authors propose that GPA4 performs a general function in

COPII vesicle assembly. How GPA4 acts in COPII vesicle formation was less clear although their results do indicate a role in this process. Overall this is an informative study although I have suggestions below to strengthen the work.

Point 1. The IP experiments in Figure 7C, 7D and 8E should include better specificity controls. It is shown in supplemental Fig S8 that the antibodies are relatively clean. However, the IP experiments in Fig. 7 should blot for other ER proteins to show membrane solubilization is efficient and that large protein aggregates are not collected in IPs. For example these same totals and IPs could be probed with antibodies for Sec12 (OsSec12b) and PDI (OsPDIL1-1), which are described in the methods.

> **RESPONSE: Thank you for your good suggestions. We have included specificity controls in our BiFC and Co-IP assays to support the specific interaction between GPA4 and OsSec23c. As suggested, antibodies for OsSec12b and OsPDIL1-1 were used to probe the IP samples to demonstrate efficient membrane solubilization in our Co-IP assays.**

Point 2. In a related specificity issue, were the C-terminal and N-terminal point mutations in GPA4 that prevented localization of GPA4 to Golgi-associated ERES tested for Sec23 interactions in the two-hybrid screen? It would strengthen the study if specific point mutations in GPA4 that interfered with OsSec23 interaction in Y2H assays also altered GPA4 localization in cells.

> **RESPONSE: We have tested the interaction between the C-terminal and N-terminal point mutations of GPA4 and OsSec23. It seems that the point mutations did not affect the interaction between GPA4 and OsSec23. We failed to find such point mutations in GPA4 that interfere with its interaction with OsSec23c. To strengthen our study, we adopted the split-ubiquitin based Y2H assay and BiFC assay to strength our conclusion.**

Point 3. I would suggest more caution in interpreting the fractionation results in Figure 8. It seems possible that changes in Sar1 distribution (panels C, D) could be due to indirect consequences of the gpa4-1 mutation. Indeed, one might have expected less membrane associated Sec23c (in a Sec23-Sec24 complex) in gpa4-1 mutant membranes instead of Sar1b and Sar1c as observed? These changes observed may reflect a more global response to manage loss of GPA4 function including changes in membrane protein and lipid composition due to activation of the UPR.

> **RESPONSE: Thank you for your valuable comments. We reanalyzed the band intensities and calculated the distribution of each four fraction. The data is listed in Supplemental Table 3. Our results showed that the membrane binding activity of each protein did not change, while the distribution of OsSar1b and OsSar1c in membrane fractions was significantly changed. Further, we observed markedly change of the ERES marker AtSar1b-GFP in the gpa4-2 mutant (new Figure 8D). These results suggest that the formation and function of COPII are likely affected.**

TPC2016-00717-RA 1st Editorial decision – *revision requested* **October 3, 2016**

On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers, which we feel, if fully addressed, will further improve your interesting manuscript.

In particular, please address the concerns of reviewer #1, issues #3 and #4:

Point #3: Please describe in the methods how the immunoblots used to generate figure 7E were probed with anti-phosphorylation and anti-OsSec23 antibodies.

Point #4: Reviewer #1 raises the concern that the control blot for Supp Figure 8A is not relevant because the samples probed with preimmune anti-GPA4 were not generated by immunoprecipitation. As it is unclear from the methods, please explain how the samples for the preimmune blot were generated, and if the samples were indeed not generated by immunoprecipitation provide a relevant control blot.

--- Reviewer comments:

[Provided below along with author responses]

TPC2016-00717-RAR1 1st Revision received October 17, 2016

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

Reviewer #1

The resubmitted manuscript improved significantly by including many control data. Thus many of the data presented seems solid and worth for publishing. Unfortunately however, I think there are still some points that can be addressed or changed before publication of this work. These points are listed below:

Major points

Point 1. A work about the characterization of the mutants of the same gene is now in press as authors cited in line 504. In this work the same gene and products were described as GLUP2/GOT1B. Thus the name of the gene (and corresponding protein as well) should be written as GLUP2/GOT1B/GPA4 in this work. Also cite the GLUP2/GOT1B paper indicate above in this work.

RESPONSE: Revised as suggested. Thanks.

Point 2. Lines 338-340 and 464-466 "Interestingly, both ..." and "Our co- IP assay..." and Fig. 7D. The results of co-IP using anti- OsSar1b (Fig. 7D) can only indicate that OsSar1b can interact with both OsSec23c and GPA4. Because authors did not analyze the stoichiometry of GPA4, OsSec23c and OsSar1b in the immunoprecipitant, this result does not indicate that these three proteins are present in the same complex. This result only indicates that a fraction of OsSar1b can interact with GPA4 in the cell lysate and another fraction can interact with OsSec23c. Thus these sentence should be rephrased.

Alternatively if the author want to use the co-IP result to discuss that these three proteins make a protein complex, the analysis of the stoichiometry these proteins by quantitative immunoblotting is essential. If the result of the analysis of the stoichiometry indicates that the molar ratio of these proteins is almost identical in the immunoprecipitant, this will be the indication that these three proteins can be present in the same complex.

RESPONSE: Our Co-IP assays showed that both OsSec23c and GPA4 can be immunoprecipitated by anti-OsSar1b antibodies. It is well known that Sec23 can recruit Sar1 by direct interaction and our Y2H assay showed that OsSec23c can directly interact with OsSar1b, but no direct interaction between OsSar1b and GPA4 was detected (Supplemental Figure 7A). These observations collectively suggest that OsSec23c may serve as a bridge to bring together OsSar1b and GPA4, and thus these three proteins are likely present in the same complex(es).

Point 3. Fig7E. Usually immunoblot with different antibodies against the same protein sets cannot be done using the same membrane at the same time. However it seems that different part of a blot membrane was probed with two different antibodies, namely anti-phosphorylation and anti-OsSec23c. Because this is not a usual way, explain the detail of the method of immunoblotting how different part of a blot membrane was probed using different antibodies.

RESPONSE: After transblotting, the PVDF membrane was cut into two parts along the marker lane. Then the left part was incubated with anti-phosphorylation antibodies, while the right part was incubated with anti-OsSec23c antibodies. We described the procedure in more detail in the revised Methods section.

Point 4. Supplemental Figure 8A preimmune control blot seems inappropriate. The preimmune Anti GPA4 sera immunoblot shown in Supp Figure 8A is not a good control as immunoprecipitation sample was not used in this experiment. As authors stated in Suppl. Figure 8A legend that IP is essential to detect GPA4 by immunoblotting, the same method used for the anti-GPA4 blot should be used to prepare the samples for the control preimmune sera immunoblot.

RESPONSE: Revised as requested as shown in Supplemental Figure 8A. Thanks.

Reviewer #2:

This revised manuscript has adequately addressed my initial concerns and I find the overall study strong.

TPC2016-00717-RAR1 2nd Editorial decision – *acceptance pending* October 18, 2016

We are pleased to inform you that your paper entitled "Glutelin Precursor Accumulation4/GLUP2/GOT1B Regulates Protein Export from Endoplasmic Reticulum in Rice Endosperm Cells" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor	**November 1, 2016**