Chloroplast Outer Membrane β-Barrel Proteins Use Components of the General Import Apparatus

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Review timeline:

TPC2019-00001-RA	Submission received:	January 1, 2019
	1st Decision:	February 19, 2019 *revision requested*
TPC2019-00001-RAR1	1st Revision received:	April 22, 2019
	2nd Decision:	May 8, 2019 *accept with minor revision*
TPC2019-00001-RAR2	2nd Revision received:	May 17, 2019
	3rd Decision:	May 22, 2019 *acceptance pending, sent to science editor*
	Final acceptance:	June 15, 2019
	Advance publication:	June 19, 2019

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.)

TPC2019-00001-RA 1st Editorial decision – *revision requested* **February 19, 2019**

We have received reviews of your manuscript entitled "Chloroplast Outer Membrane β-Barrel Proteins Use Components of the General Import Apparatus." Thank you for submitting your best work to The Plant Cell. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

We ask you to pay attention to the following points in preparing your revision, and prepare a point by point response to each of the author comments.

All three reviewers recognized the importance of the work in this manuscript. At the same time they raised few minor comments as you can see from the reviewers' reports. We think that these comments would be valuable to improve your manuscript. Please pay attention to "In view of the fact that at least one other OEP does not engage the Toc complex but OEP24 seems to do just that, at least one more additional protein of the OEP family needs to be studied regarding its import behavior to ensure that this is not a phenomenon specific for OEP24."

-Supplemental materials should be restricted to large datasets and tables, presentation of replicates, and validation of reagents, methods, or genotypes. Any data that are used to support the major claims must be in the main manuscript. Supplemental figure legends must indicate what figure in the main manuscript is supported by the supplemental data presented. Please justify how each of the supplemental figures meet the criteria.

-Sampling methods and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc.), along with a clear description of and rationale for any statistical analyses conducted. The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

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

TPC2019-00001-RAR1 1st Revision received April 22, 2019

Reviewer comments and **author responses**:

Reviewer #1:

The authors address the question of how a conserved beta barrel protein in the outer envelope of plastids, OEP80, is imported/inserted into the membrane. The general process of beta barrel insertion into the OE of chloroplasts has long been enigmatic and certainly merits publication in TPC.

For Toc75, another member of this family, it was shown that is travels by using at least parts of the general import pathway depending on a bipartite transit peptide containing a glycine rich region, which is missing in OEP80.
For other OE proteins predicted to share the beta barrel structure, there are only few data available, though for at least one it was demonstrated that it needs neither a receptor at the chloroplast surface nor any ATP for its integration. No data on competition with pSSU are available.

In the present manuscript the authors show that OEP 80 is N-terminally processed, indicating it features a cleavable transit peptide, and that it engages Toc159 as well as Toc75 during translocation. As a control they used OEP24, a solute channel, which import had not been analyzed before and turns out not to have such a transit peptide, though it might interact with Toc75 during import. A chimeric protein consisting of the N-terminus including the POTRA domain, however, behaves like OEP80, but only in the presence of the OEP80 transit peptide. The authors conclude that the POTRA domain is the reason for Toc75 and OEP80 featuring an essential TP, whereas OEP24 does not seem to need one.

The methodology applied, though not innovative, is sound and convincing and the manuscript is very well written.

I have, however, a few issues that need to be addressed.

Point 1. In view of the fact that at least one other OEP does not engage the Toc complex but OEP24 seems to do just that, at least one more additional protein of the OEP family needs to be studied regarding its import behavior to ensure that this is not a phenomenon specific for OEP24.

Co-IP data nicely show that OEP80 can be pulled down with Toc159 and Toc75, indicating that one acts as a receptor and the other might help with the actual membrane integration. For OEP24 there are only data for Toc75 and I wonder why. It would be crucial for understanding the whole process to investigate if OEP24 also interacts with a receptor or rather behaves like other OEPs and integrates independently of surface exposed receptor proteins.

> **RESPONSE: Reviewer 1 asked that we check if OEP24-T7 pulled down Toc159. We had not checked this previously but included this blot when repeating the pulldowns with OEP40-T7. Interestingly, while both OEP24-T7 and OEP40-T7 pulled down Toc75, neither pulled down Toc159. This blot is included in the new Figure 6 panel B. We added a sentence to the results section to describe this (lines 296-299) and the legend (line 779).**

Point 2. Very recently, the Schleiff group published that targeting and integration of OEP24 is critically dependent on the composition of the penultimate beta strand. I am aware that this publication was not available upon submission of the present manuscript, but now this needs to be included into the discussion.

> **RESPONSE: Reviewer 1 asked that we address a recent study in the discussion. This was added to the end of the first paragraph of the discussion section (lines 320-328). A citation of this study was added to the references section.**

Reviewer #2:

Excluding Toc75, other chloroplast OEM β-barrel proteins, OEP24, OEP37, and Toc75-V/OEP80, are also predicted to have a transit peptide at their N-terminal end, whereas OEP23 and TGD4 do not. In the case of Toc75-V/OEP80 in Arabidopsis, the approximately 52 amino acid residues of the N-terminal are dispensable in the targeting, insertion,

or functionality of this protein. However, whether the predicted transit peptides in the other proteins are involved in chloroplast targeting has not yet been experimentally confirmed. The studies of the targeting and insertion mechanism of OEM β-barrel proteins are still an unknown world. Day and colleagues have demonstrated that the N terminus of OEP80 behaves like a canonical transit peptide. Unlike Toc75, OEP80's N terminus is not responsible for envelope sorting. Instead, the C terminal transmembrane is required to prevent full translocation into the stroma. They also show that the reason OEP80 requires a transit peptide while most other OM β-barrels do not is due to its intermembrane space localized POTRA domains. Moreover, they also suggest that both OM β-barrels with or without transit peptides use components of the general import apparatus.

The findings in this manuscript are novel and very interesting both to researchers in the chloroplast field but also for researchers of the broader protein transport community. I have only a few modest concerns and comments.

Point 1. In Figure 1C, the authors suggested that OEP80 ortholog from Pisum sativum (pea) is also processed in vitro chloroplast import assay. However, the translation product input (TL) line also contained translation products of mature size (maybe produced by methionine downstream of the initial start codon). Unfortunately, it is the same size as the actual mature form, causing confusion. In addition, the ratio of the precursor form to the mature form of the T line is very similar to that of the TL line.

RESPONSE: In their first point, reviewer 2 claim that a band the size of the mature PsOEP80 is present in the PsOEP80 translation product. We cannot see this band, so we do not believe there is a problem.

Point 2. In Figure 2 and Figure 5, why did not the amount of precursor form of OEP80 and Toc75 increase in import inhibition or unprocessed conditions by treatment of competitor or boiled Plsp? Moreover, it is necessary to include statistical analysis to ensure the reliability of data.

RESPONSE: In their 2nd point, reviewer 2 takes issue with the fact that the amount of precursor of Toc75 and OEP80 does not change between treatments in figures 2 and 5. The processing and import of OEP80 is very inefficient, so the small amount of precursor converted into mature does not cause a noticeable impact the amount of precursor. The same can be said of Toc75 in Figure 2. As for Toc75 in figure 5, the original study that investigated targeting of Toc75 (Tranel et al., 1995) also did not see a large increase in the precursor form during competition assays.

In their 2nd point, reviewer 2 also asks for statistical analysis of the import competition assays. The repetitions for these assays were performed with some differences (competitor concentration, import time, denaturant used for recombinant protein), so they are not suitable for statistical analysis.

Point 3. In Figure 2-panel C, why is the precursor form of RSSU detected in mRSSU treatment conditions? This problem is closely related to the reliability of the data.

RESPONSE: In reviewer 3's third point, they ask why the precursor form of RSSU is detected in the mRSSU treatment conditions. This is because there are 2 types of RSSU in this experiment. First there is the recombinant RSSU which is either prRSSU or mRSSU and determines the treatment. Then there is the radiolabeled RSSU, which is only the prRSSU form. This was included as a control to show that prRSSU would compete with other prSSU. The radiolabeled prRSSU was subjected to all treatments, including those with no recombinant protein.

Point 4. Page 12 L227~L229, "When 80TP was removed, the resulting protein associated with the chloroplasts but was degraded by both proteases (Figure 4C, D construct 2), demonstrating that the YFP moiety was not taken across the OM." However, the authors performed experiments that only treated one protease (thermolysin).

RESPONSE: In their 4th point, reviewer 2 identified an error in the text discussing figure 4 where we said construct 2 was degraded by both proteases (thermolysin and trypsin). We did not include construct 2 in trypsin results (Figure 4D), so we changed "both proteases" to "thermolysin" at line 238. This did not affect our conclusions, since degradation by thermolysin is sufficient to indicate the protein was outside of the chloroplast.

Point 5. In Figure 4 and 6, the authors transiently expressed constructs tagged with the YFP and then analyzed their localization or performed pull-down assay in isolated chloroplasts. In addition to fractionation and pull-down assay results, the author should provide fluorescence microscope image. The manuscript would be strengthened if it could be provided.

RESPONSE: In their 5th point, reviewer 2 suggests that we include fluorescence microscopy images of tissue expressing YFP tagged proteins. We opted not to do this because we felt that this opened a new can of worms unnecessarily. The fact that GFP is fluorescent had nothing to do with our selection of its use in our experiments. Rather it was chosen out of convenience as a passenger protein. Additionally, fluorescence microscopy does not give the required resolution to determine the location of the GFP within the chloroplast system, and so no new information would be forthcoming beyond what we have already shown by the protease treatments and western blots, which we believe are sufficient to support our conclusions.

Point 6. In Figure 5-panel B, there is no significant difference in migration of precursor form and mature form of PsOEP80 compared to other images. Is this really a mature form?

RESPONSE: In their 6th point, reviewer 2 suggest that the migration of the precursor and mature forms of PsOEP80 are not different enough to distinguish. While the band representing the mature form is very weak and the difference in size is small, we believe they are distinct. More importantly, both the precursor and mature PsOEP80 decrease in intensity when competing substrate is added.

Point 7. In Figure 6, the amount of input for each treatment (expression) is very different. For accurate analysis, it is required to perform experiments using the same amount of input.

RESPONSE: In their 7th point, reviewer 2 suggests that we should have normalized input to the amount of tagged protein. Instead, we normalized input to chlorophyll concentration as a proxy for total concentration of TOC components. We believed our normalization would give more accurate results, because we are asking if TOC components are pulled down. In an ideal world, the ratio of tagged protein to TOC components would be the same for each construct, but we have very little control over the expression/accumulation of the tagged constructs. Also, control construct X is not targeted to the chloroplast, so we could not normalize with this since it is not present in the solubilized chloroplast input.

Reviewer #3:

This manuscript is a carefully done work to show the presence of an N-terminal transit peptide on OEP80, a chloroplast outer envelope beta-barrel protein of the OMP85 family. Prior work has shown that Toc75, a chloroplast outer envelope OMP85 family beta barrel, has a unique import pathway in that it requires a cleavable, bipartite N-terminal transit peptide containing a stromal targeting sequence followed by a glycine-rich containing signal required for envelope targeting. Toc75, like other OMP85 proteins, contains N-terminal POTRA (polypeptide translocation associated) domains followed by a C-terminal transmembrane beta-barrel. There is another OMP85 family member, OEP80 also with POTRA domains, in the chloroplast outer envelope, that was shown to undergo processing during in vitro import thus suggesting the presence of a cleavable, transit peptide; however, this protein lacks a discernible envelope sorting signal. Other outer envelope beta-barrels lack cleavable transit peptides for their localization to the chloroplast outer envelope. The authors seek to characterize the requirement of the N-terminal transit peptide by beta-barrel proteins of the outer envelope such as OEP80 and by analogy Toc75.

Through the data presented, the authors clearly demonstrate the role of the cleavable, N-terminal targeting sequence as being required for import of the intermembrane space localized, soluble POTRA domains of OEP80, while having little or no effect on the import of the beta-barrel portion. Overall, I found this manuscript to contain carefully done experiments and was largely well-written (see below).

The authors use OEP24 as an example of a beta-barrel protein that does not require an N-terminal, cleavable sequence for targeting and import. However, if OEP24 has POTRA domains appended to the N terminus, it loses the ability to successfully localize to the outer envelope in the absence of a transit peptide. They also show that if OEP80 has it's POTRA domains removed, it no longer requires the transit peptide for outer envelope localization. These experiments strongly suggest the need for a transit peptide to get the POTRA domains across the outer envelope.

Not only does OEP80 require a transit peptide, like Toc75, but it also uses the Toc import pore, which was nicely shown by the competition assays and pulldown experiments.

Overall, this is a well-done study.

I have a few minor issues as described below.

Point 1. Lines 90-108: The authors need to be more nuanced in this part of the Introduction. It is fairly well established that OMP85s did not change orientation during evolution as was presented in the paper by Sommer et al. (2011). Chen et al. (2016) and Paila et al. (2016) both clearly show the topology of Toc75 was maintained evolutionarily from cyanobacterial or eubacterial OMP85. What is not known, however, is how beta-barrel OEPs lacking POTRA domains are inserted into the outer envelope.

RESPONSE: Review 3 asked that we revise our paragraph in the introduction where we discussed the study by Sommer et al. (2011). We made substantial changes to this section to prevent the misunderstanding that we agree with the findings of Sommer et al. (2011). We did not completely remove discussion of Sommer et al. (2011) because it is important for establishing the significance of our study. When referring to the later studies by Chen et al. (2016) and Paila et al. (2016), we change the word "suggested" to "established" at line 120 to ensure the reader understands that we accept the findings of the more recent studies.

Point 2. Lines 110-112: The authors use "intermembrane space" in reference to the location of POTRA domains as shown by Sommer, et al. (2011). Don't you mean the cytoplasmic face of chloroplasts? As written it seems counter to what the referenced articles actually showed. Chen, et al., (2016) and Paila, et al. (2016) both clearly showed that the POTRA domains were located in the IMS; whereas Sommer, et al., (2011) suggested the POTRA domains were on the cytoplasmic face.

RESPONSE: Review 3 pointed out that at lines 110-112 we wrote "intermembrane space" when we meant "cytosol." This has been corrected at line 120.

Point 3. Please be more consistent throughout when referring to various orthologs of OEP80. The figures mostly do a good job of labeling proteins as AtOEP80 versus PsOEP80 or HvOEP80, but the text often lacks those distinctions making for some confusion.

RESPONSE: Reviewer 3 asked that we make it clear which OEP80 orthologs we are referring to throughout the text. Changes were made at lines 142, 143, 145, 150, 168, 169, 173, 177, 186, 190, 193, 201, 205, 209, 223, 226, 229, 248, 261, 280, and 284.

Point 4. Some of the gel images would benefit from indicators to orient the reader to what is being presented. For example, in Fig. 1, you indicate the precursor and mature forms, but what are the other bands in several of the images? Figure 4C-D could also benefit from additional labels indicating what proteins the bands represent.

RESPONSE: Review 3 asked that we include indicators in some figures near bands of unclear identity. These were added to figures 1, 3, and 4.

TPC2019-00001-RAR1 2nd Editorial decision – *accept with minor revision* May 8, 2019

We have received reviews of your manuscript entitled "Chloroplast Outer Membrane β-Barrel Proteins Use Components of the General Import Apparatus." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following:

As you can see below, the reviewer #2 made a comment on Figures 2 and 5. Please address this point either experimentally or by discussion.

TPC2019-00001-RAR2 2nd Revision received May 17, 2019

Reviewer comments and **author responses**:

Reviewer #1:

The authors addressed my points (and most of those of the other two reviewers as I saw) very adequately. Though the data set would have been even more convincing with OEP40 import I am aware that some proteins simply don't do in vitro import in spite of their being bona fide plastid constituents. Thus, I have no more issues with the manuscript being published in its revised form.

Reviewer #2:

In response to reviewers, the authors also know that the processing and import of OEP80 are very inefficient, and the results of this type of experiment are always likely to include some variations.

Point 1. Although these experiments were performed with some differences, statistical analysis based on the results of repeated experiments on each panel in Figure 2 and 5 is necessary to ensure the reliability of the data. At least the experiments in each panel would have been performed under the same conditions.

RESPONSE: As we stated earlier when we returned the revision, the repetitions for these experiments were not identical (different time points, different constructs), and so statistics were not possible. Nonetheless, each repetition experiment showed the claimed competition. Rather than redo these time-consuming experiments three times identically to generate the statistics, we have opted to publish the repetitions in Supplemental Figure 3. We believe that anyone looking at these figures will agree that we have demonstrated competition between prOEP80 and prSSU for processing and for targeting.

Reviewer #3:

I have carefully reviewed the revised manuscript and the authors have addressed my concerns.

TPC2019-00001-RAR2 3rd Editorial decision – *acceptance pending* **May 22, 2019**

We are pleased to inform you that your paper entitled "Chloroplast Outer Membrane β-Barrel Proteins Use Components of the General Import Apparatus" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor **June 15, 2019**