An evolutionarily distinct chaperone promotes 20S proteasome α-ring assembly in plants

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MS TITLE: An evolutionarily distinct chaperone promotes 20S proteasome α-ring assembly in plants

AUTHORS: Richard S. Marshall, David C. Gemperline, Fionn McLoughlin, Adam J. Book, Kay Hofmann, and Richard D. Vierstra
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns by editorial revision of the paper. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

A previously unknown variant of a 20S proteasome assembly factor, PBAC5, is characterized in Arabidopsis. It is related to the conserved PBAC1 and PBAC2, which in other species such as humans form a heterodimer. PBAC5 is widespread in plants but is found only sporadically outside this group. The authors show that PBAC5 associates with PBAC1 and a subset of alpha subunits of the 20S proteasome, that a HbYX motif at its C-terminus is important for its function in plants (but not yeast), that PBAC5 promotes 20S assembly in Arabidopsis, and that loss of PBAC5 results in proteotoxic stress sensitivity. The work is thorough and carefully done. It is a substantial addition to the plant proteasome assembly field.

Comments for the author

Marshall et al. characterize PBAC5, an Arabidopsis 20S proteasome assembly factor that has diverged from the PBAC1/PBAC2 (Pba1/Pba2; PAC1/PAC2) family of heterodimeric assembly factors. It is found throughout the Planta supergroup (but maybe not red algae?), but only sporadically elsewhere in other taxa. PBAC5 also has a HbYX sequence at its C-terminus, like the Pba1/2 assembly factors where it is known to be essential for proteasome precursor binding. The authors show that PBAC5 functions in 20S proteasome assembly, associates with PBAC1 and certain 20S alpha subunits, and while not required for viability, is required for proteotoxic stress resistance in Arabidopsis seedlings. Interestingly, they also were able to show that Arabidopsis PBAC1 and PBAC2 cannot complement an S. cerevisiae strain deleted for the orthological PBA1 and PBA2 genes, but if PBAC5 is added, the three plant genes together can complement the yeast mutant. This does not require the PBAC5 HbYX motif, while in plants all three HbYX motifs are needed.

Overall, this study very clearly shows that PBAC5 is a variant 20S proteasome assembly factor. The data are of high quality, and the experiments are well controlled. More insight into the mechanism could be provided, however. With the addition of a few results, I would be happy to recommend this for publication in the Journal of Cell Science. The key experiments I'd like to see are the following:

1. The authors claim that PBAC5 forms part of a heterotrimer with PBAC1 and PBAC2. However, there is no direct evidence for this trimeric complex. I would like to see the authors show this by coexpressing the three proteins in E. coli or expressing them individually and mixing extracts or purified proteins and then using SEC or gradient fractionation to show the three proteins forming a complex. It might even be possible to show the proposed PBAC1/2/5-alpha4/5/6/7 complex this way, but I would not require that.
2. This relates to the preceding concern. As it stands, PBAC5 might be promoting PBAC1 stability or association with PBAC2 (a chaperone for a chaperone). Even though the authors have tagged all three subunits (PBAC1,2,5), they never show levels of the subunits by immunoblotting. The easiest place to look at this would in their yeast analysis. I'd like to see the levels of all three (tagged) proteins analyzed, with a particular interest in levels of PBAC1 and PBAC2 in the mutant yeast cells with and without coexpression of PBAC5.

Minor comments:

1. Note on the cartoon in Fig. 8B: Ump1 is shown on the same side of the alpha ring as the PBAC1/2/5 proteins. Granted it is largely disordered, but Ump1 is mostly on the inside of the precursor ring (with several beta subunits) while the PBAC proteins would be on the outside of the ring.
2. P. 9: Please define NAC53 and NAC78
3. Fig. 6: What is going on with Rpn5? Does it form a smaller intermediate (not full RP)? It consistently migrates at a later part of the gradient than the other RP subunits.

Reviewer 2

Advance summary and potential significance to field

JOCES/2020/249862

This work is a thorough and very interesting analysis of an evolutionarily distinct chaperone of proteasomal alpha ring assembly in Arabidopsis. Congratulation to the authors for their outstanding manuscript which was most likely written during the CoV2 lockdown. The manuscript is a real pleasure to read, will be a valuable contribution to the ubiquitin proteasome filed and is suited for publication in JOCES.

Comments for the author

I have only minor points which should be addressed for amendment:

The reconstitution of cellular fluorescence was yielded by PBAC5 and PBAC1 co-expression but not with PBAC2 co-expression. I am a bit confused. What is the authors' strongest argument for a 1-2-5 trimer ? Is it possible that two functionally interchangeable 1-2 and 1-5 dimers exist in Arabidopsis ? Please clarify.

Lanes 372-376: HA-PBAC5 is found in the top three fractions of the glycerol gradient without any a subunit. I would expect an early a subunit ring intermediate with PBACs in the top fractions as stated in lane 373. Maybe these intermediates could be trapped by addition of MG132, but I see none in the presented figure.
Instead HA-PBAC5 delta HbYX is in the top fraction co-migrating with a subunits. The authors claim that the mutant protein is precluded from a subunit association. If HA-PBAC5 delta HbYX interacts with PBAC1, it could still be associated with a subunits. Please reconsider your statements in lines 372-376.

Do you happen to have tagged versions of PBAC1 and 2 to test interaction with HA-PBAC5 or vice versa ? Co-IPs should be added to Fig. 2A. TAP-PBAC5 purification could be an alternative to test interaction with PBAC1 and 2.

Abstract line 40 either a template or templates
Line 93: I would say 2 x 32 subunits instead of 64

Fig. 5D out of curiosity: what is the origin of Ub2, Ub3 and Ub4 without substrate, are these left overs from proteasomal degradation ?

First revision

Author response to reviewers' comments

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August 25th 2020

Dear Editors,

Please find attached a revised version of our manuscript (JOCES/2020/249862) entitled “An evolutionarily distinct chaperone promotes 20S proteasome α-ring assembly in plants” by Richard S. Marshall, David C. Gemperline, Fionn McLoughlin, Adam J. Book, Kay Hofmann and Richard D. Vierstra, which we re-submit for potential publication in the Journal of Cell Science. All authors have approved submission of this work. We thank both the Editor and Reviewers for the time taken in considering our manuscript. Needless to say, we were pleased to read such positive comments on the work, and hope that this revised manuscript is now acceptable for publication.

Below please find a point-by-point response to the issues raised by the Reviewers, outlining how we have made the requested changes. As you will read, we feel that we have addressed all the concerns, including the addition of several new experiments described in Figures 2D, 2E and 3C. We have also made other slight modifcations to the text in order to remain within the 8,000 word limit for the Text and Figure Legends. The main areas of the text modified to address the Reviewer’s comments are highlighted in yellow in the revised manuscript.

Comments from the Reviewers are shown in bold italics; our responses are shown in regular font:

Reviewer #1:

Overall, this study very clearly shows that PBAC5 is a variant 20S proteasome assembly factor. The data are of high quality, and the experiments are well controlled. More insight into the mechanism could be provided, however. With the addition of a few results, I would be happy to recommend this for publication in the Journal of Cell Science.

We thank Reviewer #1 for his/her positive comments about our work.

The key experiments I’d like to see are the following:

1. The authors claim that PBAC5 forms part of a heterotrimer with PBAC1 and PBAC2. However, there is no direct evidence for this trimeric complex. I would like to see the authors show this by co-expressing the three proteins in E. coli or expressing them individually and mixing extracts or purified proteins and then using SEC or gradient fractionation to show the three proteins forming a complex. It might even be possible to show the proposed PBAC1/2/5-α4/5/6/7 complex this way, but I would not require that.

We thank Reviewer #1 for this important point, as indeed our previous interaction studies did not provide sufficient evidence for a trimeric PBAC5-PBAC1-PBAC2 complex. To address this point, we have undertaken two separate but related approaches to directly study the binding of the three PBAC-type chaperones, as now described on lines 260-270. For the first approach, we performed co-immunoprecipitation assays with the chaperones using our previously generated doa5-1 Δpba1 Δpba2 yeast lines expressing tagged forms of the chaperones in various combinations (now shown in a new Figure 3C). Consistent with a trimeric complex, we observed that when HA-PBAC5 was immunoprecipitated, PBAC2 was co-immunoprecipitated only when PBAC1 was also present. Similarly, when myc-PBAC2 was immunoprecipitated, PBAC5 was co-immunoprecipitated only when PBAC1 was again present. Finally, PBAC1 immunoprecipitation resulted in the detection of both PBAC5 and PBAC2.

Given that endogenous yeast proteins (proteasome components or chaperones) might have artifactually contributed to the co-immunoprecipitation studies, we then generated a recombinant approach, now shown in new Figures 2D and 2E. Here, we expressed and purified tagged versions of PBAC1, PBAC2 and PBAC5 from Escherichia coli, and then performed in vitro pulldown experiments to assess the interaction between PBAC5 and PBAC2 in the presence and absence of PBAC1. Again, we observed that PBAC5 and PBAC2 did not interact when mixed together in the absence of PBAC1, but did associate when PBAC1 was included.

Taken together, we hope that these two approaches are sufficient to convince Reviewer #1 that a trimeric PBAC5-PBAC1-PBAC2 complex assembles both in vitro and heterologously in vivo, with PBAC1 being the critical tether that connects PBAC5 and PBAC2.

2. This relates to the preceding concern. As it stands, PBAC5 might be promoting PBAC1 stability or association with PBAC2 (a chaperone for a chaperone). Even though the authors have tagged all three subunits (PBAC1,2,5), they never show levels of the subunits by immunoblotting. The easiest place to look at this would in their yeast analysis. I'd like to see the levels of all three (tagged) proteins analyzed, with a particular interest in levels of PBAC1 and PBAC2 in the mutant yeast cells with and without co-expression of PBAC5.

We thank Reviewer #1 for this suggestion. As part of the experiment described above, we immunoblotted total yeast protein extracts with anti-FLAG, anti-HA and anti-myc antibodies to determine the levels of these proteins in yeast strains expressing different combinations of the chaperones. As Reviewer #1 will observe (see the "Input" lanes on the left of new Figure 3C), there appears to be little change in the levels of the chaperones regardless of whether they are expressed only in pairs, or all three of them together. We do however point out to Reviewer #1 that this experiment is slightly artificial, for two main reasons: i) these chaperones are expressed from the constitutive GPD1 promoter, and therefore their levels may be artificially high; and ii) this experiment was performed in cells containing the doa5-1 mutation, which impairs proteasome activity, and hence might slow turnover. Further experiments will thus likely be necessary to fully understand the accumulation and/or degradation of these chaperones.

Minor comments:

1. Note on the cartoon in Fig. 8B: Ump1 is shown on the same side of the alpha ring as the PBAC1/2/5 proteins. Granted it is largely disordered, but Ump1 is mostly on the inside of the precursor ring (with several beta subunits) while the PBAC proteins would be on the outside of the ring.

We thank Reviewer #1 for noticing this error, and have now corrected the model accordingly, showing UMP1 to be located more towards the inside of the α-ring where the β-subunits become incorporated.

2. Page 9: Please define NAC53 and NAC78

The term NAC has now been defined in the text on line 284 as requested.

3. Figure 6: What is going on with Rpn5? Does it form a smaller intermediate (not full RP)? It consistently migrates at a later part of the gradient than the other RP subunits.

The Reviewer raises a very interesting point. During many glycerol gradient analyses performed over a number of years in the Vierstra lab, we have consistently noticed that RPN5 migrates differently than all other proteasome RP subunits that we have analyzed (see, for example, Book et al., 2009; Book et al., 2010; Lee et al. 2011; Marshall et al., 2015; Gemperline et al., 2019). While we do not know the reason for this in Arabidopsis, we note that Rpn5 is an unusual proteasome subunit in yeast, because it can also assemble as part of the COP9/signalosome complex in addition to the RP (Yu et al., 2011). We therefore speculate on lines 386-389 that the same might be true for the Arabidopsis protein, which could cause the unusual migration pattern, stating:

"We note that RPN5, but not the other Arabidopsis RPN subunits tested, also consistently sedimented with a distinct species, which was slightly smaller than free RP (Fig. 6). The nature of this particle is unknown but could reflect RPN5 also integrating into the structurally related COP9/signalosome complex, as seen in yeast (Yu et al., 2011)."

Reviewer #2:

This work is a thorough and very interesting analysis of an evolutionarily distinct chaperone of proteasomal alpha ring assembly in Arabidopsis. Congratulation to the authors for their outstanding manuscript, which was most likely written during the CoViD2 lockdown. The manuscript is a real

pleasure to read, will be a valuable contribution to the ubiquitin proteasome filed and is suited for publication in JOCES.

We thank Reviewer #2 for his/her positive comments about our work.

I have only minor points which should be addressed for amendment:

The reconstitution of cellular fluorescence was yielded by PBAC5 and PBAC1 co-expression but not with PBAC2 co-expression. I am a bit confused. What is the authors' strongest argument for a 1-2-5 trimer? Is it possible that two functionally interchangeable 1-2 and 1-5 dimers exist in Arabidopsis? Please clarify.

We thank Reviewer #2 for this point, which was also raised by Reviewer #1. Both Reviewers are correct that the earlier version of our manuscript did not provide direct evidence for a trimeric PBAC5-PBAC1-PBAC2 chaperone complex. However, as described in our response to Reviewer #1, we now provide two additional lines of evidence in the form of in vitro binding assays using chaperones expressed and purified from E. coli (new Figures 2D and 2E), and co-immunoprecipitation of the chaperones from yeast cells (new Figure 3C) that we believe provide compelling evidence for a trimeric chaperone complex anchored by PBAC1 which binds to both PBAC2 and PBAC5.

Lines 372-376: HA-PBAC5 is found in the top three fractions of the glycerol gradient without any α-subunit. I would expect an early α-subunit ring intermediate with PBACs in the top fractions as stated in lane 373. Maybe these intermediates could be trapped by addition of MG132, but I see none in the presented figure. Instead HA-PBAC5ΔHbYX is in the top fraction co-migrating with α-subunits. The authors claim that the mutant protein is precluded from α-subunit association. If HA-PBAC5ΔHbYX interacts with PBAC1, it could still be associated with α-subunits. Please reconsider your statements in lines 372-376.

Reviewer #2 is correct that our statement about the possible association of HA-PBAC5 with CP assembly intermediates in the glycerol gradients is undermined by the fact that no such intermediates are visible when probing the fractions with CP subunit antibodies. We suspect that this is because any such assembly intermediates are present at very low levels when the chaperone complex is fully functional. We have thus modified the text on lines 380-385 to state that:

"Interestingly, the HA-PBAC5 protein was found in the top four fractions of the gradient, consistent with an association with CP assembly intermediates, which we assume are in low abundance relative to the fully assembled CP. In contrast, the HA-PBAC5(ΔHbYX) protein was almost exclusively in the top fraction, suggesting that it was either free protein precluded from associating with α-subunits without its HbYX motif, or the PBAC5-PBAC1-PBAC2 trimer alone (Fig. 6)."

We hope this is sufficient to address the concerns of Reviewer #2 on this point.

Do you happen to have tagged versions of PBAC1 and 2 to test interaction with HA-PBAC5 or vice versa? Co-IPs should be added to Fig. 2A. TAP-PBAC5 purification could be an alternative to test interaction with PBAC1 and 2.

As stated above, our manuscript now employs both in vitro pulldown and in vivo co-immunoprecipitation studies with tagged versions of the PBAC1, PBAC2 and PBAC5 chaperones to show that PBAC5 and PBAC2 both interact with PBAC1, but not with each other, in a hetero-trimeric complex (new Figures 2D, 2E, and 3C). Unfortunately, we do not currently have Arabidopsis plants expressing tagged versions of PBAC1 or PBAC2 to perform similar assays in planta. However, our manuscript does contain immunoprecipitations of HA-PBAC5 from Arabidopsis extracts followed by mass spectrometric analysis to identify interacting proteins (Figure 7). While this did not provide direct evidence for PBAC5 binding to the other PBAC chaperones, both PBAC1 and PBAC2 were abundant in the co-immunoprecipitated samples.

Abstract line 40 either a template or templates.

Thank-you for catching this error, it has been fixed as suggested.

Line 93: I would say 2 x 32 subunits instead of 64.

We agree, and now state on lines 93-95 that “Assembly of a complete 26S proteasome from its 32 or more distinct constituents is a highly complicated process that requires a suite of dedicated chaperones and maturation factors for precise construction of both the CP and RP”.

Fig. 5D out of curiosity: what is the origin of Ub2, Ub3 and Ub4 without substrate, are these left overs from proteasomal degradation?

Unfortunately we are currently unsure of the origin of the free di-ubiquitin, tri-ubiquitin and tetra-ubiquitin species, but they appear to be more abundant in plant cell extracts than they are in extracts from yeast or mammalian cells (see van Nocker and Vierstra, 1993). These species could certainly be remaining after deconjugation from proteasome substrates, or they could reflect newly synthesized polyubiquitin chains awaiting transfer to protein substrates, as they are competent in ubiquitin transfer.

We hope we have been able to fully address all the concerns of the Reviewers in a satisfactory manner, and thank you in advance for your continued consideration of our manuscript. We have also made a number of editorial changes to the manuscript in order to improve its overall readability, and to attempt to stay within the word limit. If you have any additional questions, please do not hesitate to contact us. We look forward to hearing from you.

Yours sincerely,
Dr. Richard D. Vierstra,
George and Charmaine Mallinckrodt Professor

Dr. Richard Marshall
Research Scientist

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Literature cited:

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Second decision letter

MS ID#: JOCES/2020/249862

MS TITLE: An evolutionarily distinct chaperone promotes 20S proteasome Î±-ring assembly in plants

AUTHORS: Richard S. Marshall, David C. Gemperline, Fionn McLoughlin, Adam J. Book, Kay Hofmann, and Richard D. Vierstra
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

I am happy with the additional results and the other small modifications that I had requested. In my opinion, this is ready for publication.

Comments for the author

No new suggestions

Reviewer 2

Advance summary and potential significance to field

As already stated in my review of the original submission the work is thoroughly done and suited for publication in JOCES.
Congratulations to the authors for these timely and interesting studies.

Comments for the author

I am pleased to see that the authors satisfactorily met my points in the revision.