The Inhibitor Endosidin 4 Targets SEC7 Domain-type ARF GTPase Exchange Factors and Interferes with Subcellular Trafficking in Eukaryotes

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Review timeline:

TPC2015-00516-RA	Submission received:	June 11, 2015
	1st Decision:	July 31, 2015 *manuscript declined*
TPC2018-00127-RA	Submission received:	Feb. 12, 2018
	1st Decision:	March 22, 2018 *revision requested*
TPC2018-00127-RAR1	1st Revision received:	June 29, 2018
	2nd Decision:	July 2, 2018 *acceptance pending, sent to science editor*
	Final acceptance:	July 17, 3018
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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.)

TPC2018-00127-RA 1st Editorial decision – *declined* June 11, 2015

Although the identification of ES4 as a new drug that inhibits SEC7-type ARF-GEFs would be of interest to many cell biologists, the manuscript as it stands is not of general interest and several conclusions are not sufficiently supported:

Point 1.The title of your ms states that ES4 interferes with subcellular trafficking and basal PIN polarity, however your data show clearly that neither endogenous PIN2 nor PIN2 is affected. Rather, effects are limited to the artificial situation of PIN1 misimpression under control of the PIN2 localisation.

RESPONSE: Strictly speaking, the effect on PIN1 on the PIN2 expression domain is still an effect on PIN trafficking and polarity. Also, PIN endocytic trafficking is affected in their endogenous expression domains, even though there are no obvious defects in polarity. However, we agree with the point and anyway, given the new set of biochemical data confirming that SEC7 domain ARF-GEFs are the direct targets of ES4, we shifted a bit the focus and thus also title of the manuscript to:

"Endosidin 4 inhibitor targets the SEC7 domain-type ARF-GEFs and interferes with subcellular trafficking in eukaryotes".

Otherwise, just to explain the observation that PIN1 is clearly affected in PIN2 expression domain but not in the other domains: According to all our previously published and unpublished data, PIN1 in the epidermis is the PIN polarity situation that is most sensitive to perturbations and thus it is common to see perturbations there more clearly than in the endogenous situation. This was the original point of generating such a genetic background and performing the genetic and chemical genetics screens.

Point 2. Based on indirect evidence from BFA washout experiments, you conclude that exocytic trafficking is only mildly affected. This is not in agreement with the fact that ES4 causes ER-accumulation of the trans-Golgi marker N-ST-GFP and the fact that ES4 causes aggregation of Golgi stacks and TGNs. Moreover, it is unfortunate that the

TEM analysis is limited to the rather marginal effect on MVB number and size.

RESPONSE: As we showed, ES4 does, to a different extent depending on the concentration, affect many intracellular trafficking routes, including endocytosis, recycling, and vacuolar delivery (Figure 3), which is consistent with the effects on the Golgi marker N-ST-GFP (Figure 3I and 3J). Endocytosis, assessed by BFA body formation and FM uptake (Figure 2A to 2F), is significantly affected. While exocytosis, assessed by BFA wash out experiments (Supplemental Figure 3K to 3R), was relatively less affected than endocytosis. Now, after clarifying experimentally that ES4 inhibits the ARF-GEFs including those that are targeted by BFA, we understand this outcome. That means that in our wash-out experiment, after removal of BFA, ES4 still continues to block the activity of the ARF-GEFs, and thus we do not see much difference. We discuss this issue now in the revised manuscript.

In the TEM experiment, we have also observed higher number of small vesicles in a close proximity to the TGN after 2 h of 41 µM ES4 treatment, when compared to the mock treatment. We added this new observation to the revised version of the manuscript (Supplemental Figure 3T to 3U).

Point 3. The conclusion that a point mutation in d-COPI is responsible for ES4 resistance is weak. Resistance of the recapitulation line is limited to the lowest concentration of ES4, potentially because expression under the control of RPS5Ap is not suitable. Controls including overexertion of the wt-protein and the mutated protein under the control of the endogenous promoter would be required.

RESPONSE: We appreciate the comment but would like to point out that results presented in the original manuscript in fact are showing significant resistance of *es4r1* and RPS5::*es4r1* to the ES4 compound at both assessed concentrations (original/ previous Figure 4V). At 17 µM, the bar representing the primary root length of the control (PIN1-GFP) is visible but significantly lower and at 25 µM, the bar is at minimum and therefore not visible on the graph.

Following the suggestion of the reviewers, we generated and performed the analysis of the native promoter lines. *ES4R1::ES4R1* in the WT background shows sensitivity to ES4 comparable to WT, indicating the inability of the additional copy of the non-mutated ES4R1 to confer resistance to the chemical. In contrast, *ES4R1::es4r1* in WT shows significant resistance to the compound in comparison to control, albeit lower than *es4r1*, presumably due to the presence of the WT copy of the *ES4* gene. These results are comparable to those using the converse transgenic line, namely - *ES4R1::ES4R1* in the *es4r1* genetic background, in which *es4r1*-conveyed resistance is diminished by the transformed non-mutated version of ES4R1. Altogether, these results solidify the causal link between the *es4r1* mutation and resistance to the ES4 compound. However, for the revised version, we obtained important biochemical data confirming that ES4 directly targets the ARF-GEFs, and thus, we have replaced the mutant screen part with the discussion of biochemistry.

Point 4. You do not show data demonstrating that PIN1 trafficking is restored in the *esr4* mutant, so the effects of d-COP1, ARF1 activation and PIN1 trafficking remain disconnected.

RESPONSE: We appreciate the comment but would like to point out that in the original manuscript (see previous version of the manuscript Figure 4I to 4K), we describe how PIN1-GFP trafficking is restored in the *es4r1* mutant, as visualized by the formation of BFA bodies during ES4 and BFA co-treatment. Similarly supportive results were presented in the previous Supplemental Figure 4E to 4H for the RPS5A::*es4r1* transgenic lines (WT genetic background). Note that we omitted those figures in the new, streamlined version of the manuscript to focus on the new biochemical data (DARTs assay) showing that ES4 directly targets ARF-GEFs (more elaboration below).

Point 5. Mechanistic insight into the action of ES4 is missing. Given that d-COPI and the SEC7 ARF-GEFs are well-studied proteins, a mode of action should at least be discussed.

RESPONSE: Yes, we agree and we were aware of this shortcoming. We made a substantial effort and improved this aspect of the manuscript. We have identified ARF-GEFs as being direct targets of ES4. Our experimental data, along with docking simulations, strongly suggest that ES4 is, similar to Brefeldin A, an inhibitor of ARF-GEF function, with different substrate specificities as compared to BFA, meaning that ES4 also binds to and inhibits SEC7-containing ARF-GEFs that are insensitive to BFA.

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

[Reviewer comments shown below along with author responses]

TPC2018-00127-RA Submission received Feb. 12, 2018

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

Reviewer #1:

In this study, the authors discovered a novel compound that modifies the subcellular localization of ectopically expressed PIN1 in root epidermal cells. This compound, ES4, affected multiple trafficking pathways, including endocytic, exocytic, and vacuolar transport pathways, probably through compromising the activation of ARF GTPase mediated by a protein family with a conserved catalytic domain, the Sec7 domain. Furthermore, the authors identified a mutant that exhibited resistance to ES4 and identified the d-COPI gene as a responsible gene for this mutation.

In this work, the authors took advantage of a chemical genetic approach to dissect the complex membrane trafficking system in plants. The results presented in this manuscript are clear and interesting, and the overall quality of the work is adequately high. This work will also provide a powerful tool to investigate plant membrane trafficking pathways governed by ARF GTPases, which will be beneficial for researchers in related fields. I am enthusiastic about this work and will support publication of this work in The Plant Cell when my concerns listed below are properly addressed.

Point 1. I would like to see some more results concerning functional relationships among ARF GTPase, BFA and ES4.

RESPONSE: Regarding the effects of ES4 on ARF GTPase, we mentioned in the original manuscript that ES4 does affect the localization of the WT ARF1 (in this manuscript see Figure 5A to 5C) but not the permanently activated GTP-bound and already membrane-associated ARF1Q71L, which does not require the ARF-GEF function (in this manuscript see Figure 5D to 5F). On the other hand, the GDP-locked ARFT31N form shows a localization similar to that of the ES4-treated ARF1 wild-type form, namely a non-membrane associated, cytosolic localization (in this manuscript see Supplemental Figure 5). ES4 also affects the localization of exchange factors for ARF GTPases, the so called ARF-GEFs such as GNOM (in this manuscript see Supplemental Figure 3X to 3Y) and GNL1 (in this manuscript see Figure 3G to 3H), data that were presented in the original version of the manuscript.

These results suggest that ES4 regulates the activity of ARF-GEFs. This is now confirmed by the new biochemical data. DARTs assays showed that ARF-GEFs are direct targets of ES4. The experimental data, along with docking simulations, strongly suggest that ES4 is, similar to Brefeldin A, an inhibitor of ARF-GEF function but without the BFA bias, meaning that ES4 also binds to and inhibits SEC7-containing ARF-GEFs that are insensitive to BFA. All these data sets, along with the proper discussion, are now included in the revised version.

Point 2. Does the *es4r1* mutant also exhibit resistance to higher concentrations of BFA?

RESPONSE: We did not notice any obvious differences between *es4r1* versus WT in terms of BFA body formation (90 minutes 25 µM BFA treatment), as visualized by anti-PIN1 and anti-PIN2 antibody staining. We can provide the data, if needed. We did not pursue this issue any further. Also, the *es4r1* data were now replaced by the biochemical analysis.

Point 3. Do higher concentrations of ES4 and BFA affect the membrane association of CLC, whose assembly should also be under the regulation of ARF GTPase? Information obtained from these experiments will delineate modes of action of the chemicals more clearly.

RESPONSE: Indeed, in the original manuscript (see original/previous Figure 3E to 3F), we mentioned that CLC2-GFP is less associated with the PM after treatment with ES4 [17 µM]. Following the reviewer's enquiry, we performed a 50 µM 90 min. BFA treatment on the CLC2-GFP line, and its signal (as expected) aggregated in the cytoplasm but was also less associated with the PM in comparison to the non-treated control. We included these data and the discussion in the revised version of the manuscript (Supplemental Figure 3Z to 3Z').

Point 4. How the mutation in d-COPI confers resistance to ES4 treatment is quite interesting, which should be discussed in the manuscript more clearly. It would be helpful (and not so difficult) to see an effect of the comparable

mutation in yeast *Ret2* on the sensitivity of yeast to ES4, which will further strengthen the authors' claim on the effect of ES4 on the evolutionarily conserved system.

RESPONSE: We appreciate this valid comment and would like to point out that we mentioned in the original manuscript (in this manuscript, see Supplemental Figure 6) that the yeast strain harboring a heterozygous mutation in *Ret2* showed an unchanged sensitivity to ES4. We agree that we did not generate exactly the same type of the mutation in *Ret2*, and it will be interesting to do a follow-up study, since we now replaced the forward genetic screen with biochemical analysis.

Point 5. In the experiment presented in Figure 4, it would be better to show that overexpression of wild-type d-COPI does not confer resistance to ES4 to demonstrate that the mutation in d-COPI is actually responsible for the ES4-resistant phenotype.

RESPONSE: Following the suggestion of the reviewer, we have performed this experiment. The native promoter line ES4R1::ES4R1 in WT shows sensitivity to ES4 comparable to that of WT, indicating the inability of the additional copy of the transformed non-mutated ES4R1 to confer resistance to the chemical; similarly, plants harboring *ES4R1* expressed under the control of the *RPS5A* promoter also showed significant sensitivity, unlike the *es4r1* mutant.

Reviewer #2:

In this manuscript, Kania and coworkers describe identification of a chemical compound named endosidin 4 that affects trafficking in plant cells. The authors identified this compound initially based on its effect on the localization of PIN1:HA in PIN2:PIN1-HA;pin2 transgenic lines. They further studied the effect of ES4 on trafficking of other proteins and provide evidence that ES4 affects various intracellular trafficking pathways, including endocytosis, exocytosis, and vacuolar targeting. Moreover, the authors identified an ES4 mutant. The mutant locus encodes the ARF GEF-regulated δ subunit of coat protein I (COPI) vesicles. Chemical inhibitors such as BFA have been essential for elucidating the mechanism of protein trafficking at the cellular and molecular levels. ES4 may also be a valuable tool for elucidating the mechanism of protein trafficking at the molecular level.

Point 1. However, the authors should provide more insight into the mechanism by which ES4 affects trafficking. More mechanistic studies may be necessary to fully elucidate how this compound affects trafficking in such diverse pathways.

RESPONSE: As we have commented above (Rev. 1), the data in the original manuscript show that ARF1 and ARF-GEFs are affected by ES4 (Figure 3G to 3H; Supplemental Figure 3X to 3Y) and since these proteins are critically involved in many different trafficking processes, this explains why ES4 treatment results in a broad range of endocytic trafficking defects. In the revised version of the manuscript, we provide biochemical and docking simulation data clarifying the mode of action of ES4. ES4 acts similarly to Brefeldin A, binding to a similar part of ARF-GEFs and also targeting the BFA-resistant ARF-GEFs.

Point 2. Based on yeast experiments, the authors proposed that SEC7-domain containing ARF GEF is the target of ES4. However, the target of ES4 has not been elucidated in plant cells.

RESPONSE: In the revised version, we have included new data showing that ES4 binds to various ARF-GEFs, including the BFA-resistant ARF-GEFs (the DART experiments show increased proteolytic stability of ARF-GEF proteins as a result of ligand binding in the case of ES4) (Figure 6).

Point 3. It is not clear about the relationship between the ES4 resistant mutant and the effect of ES4 on SEC7-domain-containing GEF of Arf1.

RESPONSE: We agree with this comment. Even with the new knowledge that ES4 directly targets ARF-GEF, the connection to the *es4r1* mutant is not clear, and we therefore replaced the genetic screen data with more thorough biochemical and docking simulation analysis.

Your ms convincingly demonstrates that ES4 is an ARF-GEF inhibitor and the reported findings confirm previous work with BFA. The ms would benefit further if you could highlight conditions in which ES4 has added value compared to BFA, as it would give the manuscript a broader relevance.

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

[Provided below]

TPC2018-00127-RAR1 1st Revision received June 29, 2018

Reviewer comments on previous submission:

Reviewer #3

The manuscript by Kania et al. describes the identification of a novel chemical, Endosidin 4, which affects several instances of intracellular protein transport by specifically interfering with a subset of Sec7-domain ARF-GEFs and thus affects GDP to GTP conversion of ARFs. Importantly, they also show that membrane recruitment of one of the major ARFs, ARF1, is directly affected by ES4 treatment. The work is generally of high quality and the experiments are stringently designed. The manuscript is presented in a well-organized way and quite easy to follow. Thus, I have only a small number of remarks:

Although it does not look like there are obvious cytotoxic side effects, the authors should consider performing at least some experiments to assess this, if only for completeness' sake. For example, they could test the influence on the cytoskeleton. This could be done in the established marker lines and would therefore be relatively straightforward and quick. Since the result on ARF1 is central, but the effects at 17 µM seem rather weak judging from the micrographs in Fig.5, it would be more convincing if this could be quantified, maybe also with an additional, intermediate concentration.

Given that it seems that ARF1 is an ES4 sensitive target of certain ARF-GEFs, it should theoretically be possible to perform GEF activity assays in vitro. I do not know how difficult and time-consuming this is, but in the back-to-back-submitted manuscript by Mishev et al. such assays have been performed. I think this would further contribute to the work, however, I do not strictly insist on this experiment. I cannot comment on the quality of the docking simulations and refer this to somebody with expertise in this matter.

Reviewer #4:

In this manuscript, the authors describe Endosidin 4 (ES4) as a new inhibitory compound of the SEC7-containing ARF-GEF. The manuscript describes the initial screening strategy to identify new small molecule inhibitors of trafficking/polarity in plants. They noticed that ES4 affects intracellular trafficking pathways and that the morphology of endomembrane compartments somewhat resembled that of BFA (although without a perfect overlap). Since BFA is a known inhibitor of SEC7-domain containing ARF-GEF that has a synergic effect with ES4, the authors then tested whether ES4 could be an ARF-GEF inhibitor. This hypothesis is supported by genetic evidence (high sensitivity to ES4 of *gnl1* and *ben2* Arabidopsis mutants and sec7/SEC7 yeast strain) and DARTS-based validation. In addition, ES4 solubilizes ARF1-GFP but not a constitutive ARF1 mutant, confirming that ES4 likely inhibits ARF1 activation, which is itself regulated by ARF-GEF.

I found the demonstration that ES4 is an ARF-GEF inhibitor convincing. However, what I missed from this paper is the interest in having identified this new ARF-GEF inhibitor. As it stands, the findings for ES4 confirmed previous work with BFA, without bringing new biological insights into intracellular trafficking or polarity regulation in plants. In addition, based on what I see in the manuscript, I would not use ES4 as an alternative to BFA to inhibit intracellular trafficking or to interfere with ARF GEF function. The authors should uncover/describe a condition in which ES4 has an added value to BFA, in order to give the manuscript a broader relevance.

(The comments were addressed in a revised, highlighted version of the manuscript.)

TPC2018-00127-RAR1 2nd Editorial decision – *acceptance pending* **July 2, 2018**

We are pleased to inform you that your paper entitled "Endosidin 4 inhibitor targets the SEC7 domain-type ARF-GEFs and interferes with subcellular trafficking in eukaryotes" 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 **July 17, 2018**