Peer Review File

Manuscript Title: Biosynthesis of medicinal tropane alkaloids in yeast

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The paper is well written and excellent in design and performance of the experiments and data interpretation. I do only have minor comments as you can see in the attached file. Besides all the interesting genetic and metabolic engineering work, we must be aware that a full structural elucidation of scopolamine and hyoscyamine is not possible, because no information on chiral stereoisomers is given. It would be interesting to know, if the authors have considered chiral activity and if the amount of all products is sufficient to carry out this experiment.

Referee #2 (Remarks to the Author):

This manuscript reports a complete biosynthetic pathway for the conversion of glucose (and amino acids) to hyoscyamine and scopolamine, two FDA approved drugs belonging to the tropane alkaloyds' family of natural products, which includes several other valuable pharmaceuticals (atropine) and stimulants (cocaine). This study is a tour de force that integrates many previously known elements (the biosynthesis of different intermediate metabolites, involvement of subcellular organelles, and the final enzyme for scopolamine production) with new discoveries and technical developments that enabled this achievement. These include functional genomics to discover the hyoscyamine dehydrogenase essential to complete the pathway, identification of functional enzymes to produce PLA-glucoside (AbUGT) and littorine (DsRed-AbLS) in yeast, a novel synthetic strategy to traffic heterologous enzymes across the ER/Golgi and into the vacuole, and the demonstration that heterologous intracellular transporters (from plant) can be functional in yeast. I have no major concerns with the execution and overall conclusions of this study, and believe it is an important contribution to the advancement of plant natural product synthesis in microbes, but there are some important questions and comments that the authors need to address before it is ready for publication.

Questions:

1. The authors mention there is a putative N-terminal signal peptide in AbLS that targets the enzyme for trafficking across the ER to the vacuole in plants (Line 211). So it is confusing that fusing a GFP on the N-terminus of this enzyme would still result in it being targeted to the vacuole. Wouldn't this N-terminal fusion disrupt the functionality of the signal peptide? Does this imply that the signal does not need to be in the N-terminus? Or does it mean it is in fact not involved in this trafficking? Could it be that other internal sequence they later uncover? They need to clarify what is going on here.
2. Similarly, they mention in line 216 that the C-terminus of this enzyme is essential for stability, but they later show that they can detect enzymes with the HA-peptide fused to the C-terminus (Figs.S10, S11, and S13). They need to clarify this apparent contradiction.
3. The authors found that AbLS is N-glycosylated in yeast and in tobacco, and using site-directed mutagenesis identified the glycosylation sites and determined they are the same in both yeast and tobacco. However, AbLS is heterologous in both systems so these results do not necessarily reflect what goes on inside A. belladona. What are the glycosylation similarities between tobacco and A.belladona?

Are they assuming they are the same? Beyond the specific glycosylation sites, are the glycosylation moieties the same between both plants and between them and yeast? It seems appropriate for the authors to expand their discussion/explanations on these points (or add another supplementary note if they are lacking space). Additionally, point mutations in yeast cause incomplete or lack of glycosylation for a fraction of the enzyme (e.g. lane 5 Fig 11B) but not in tobacco (e.g. lane 7 Fig S11A). There are also clearly observable heavier (~150KDa) and lighter (~27-37KDa) bands in enzymes expressed in tobacco but not in yeast, suggesting there are fundamental differences in glycosylation and processing that are not discussed.

4. Related to 3, are the glycosylation sites important for expression, and more importantly for activity of AbLS in yeast? Did they ever test their quadruple mutant (or single mutants) for activity (in the cell)?

5. In line 159, the authors suggest that the internal SP of AbLS blocking its active site explains why the enzyme is inactive in yeast (including in different compartments). However, when they fused this enzyme to soluble fluorophores (to resolve putative TGN sorting obstruction) the problem seemed to go away. How can fusion of these fluorophores prevent the internal pro-peptide from blocking the active site? Related to question 1, how is an N-terminal fusion meant to disrupt TGN targeting still end up targetting the enzyme to the vacuole? Figure 4B indicates the enzyme still makes its way through the Golgi and into the vacuole. It is also not clear (or easy to find) what is the origin of the transmembrane domain in the engineered enzyme. Again, what happened to the pro-peptide in the final, successful, construct? I think the authors need to give a better explanation of their enzyme engineering description and rationale to clarify these questions, especially given that this is such an important part of the story (novelty and results).

6. Related to question 5, the authors attribute the effect of the different fluorophores on the activity of AbLS to their oligomeric states, with higher oligomeric states leading to higher activity. Why is oligomerization important for activity? What GFP version did they use (GFP naturally makes dimers, not monomers)?

7. Also not clear why the presence of a degradation product ~37KDa in Fig S13 leads the authors to conclude that the protein signal in AbLS is not specific to this enzyme. Couldn't it be that tobacco is able to recognize it as a peptide that needs to be processed even if it's specific to AbLS? Perhaps the term "specific" is not clear.

8. How come a 37KDa band is observed in Figs. S10 and S13, but in Fig. S11 there is a ~30KDa band instead? What is the difference between these experiments to account for this?

9. In Fig S14, DsRed-AbLS looks like it's located in the lumen of the vacuole rather than the vacuolar membrane, yet the authors conclude it is anchored to the membrane with the catalytic domain inside the lumen. What evidence do they have that this is the correct topology of the enzyme? If it's truly on the membrane, why doesn't it look more like NtJAT1-GFP (same figure) or FM4-64 in Fig 3B?

10. Also in Fig S14, it is difficult to observe localization of NtMATE2-GFP on the PM or vacuolar membrane. It looks like most of the protein is localized to the lumen of the vacuole or localized throughout the cytosol (given it's a membrane protein, this could be explained by a partially proteolyzed protein leaving soluble GFP in the cytosol). Can the authors provide additional evidence that this protein is indeed targeted to the membranes they are claiming (PM and vacuole)? Otherwise, can they make a stronger argument for why these images show what they claim they show? Otherwise they should revise their claims summarized in Fig 4B, or at least be more cautious with their language to reflect the unconvincing evidence they show for this claim in Fig S14.

11. In the abstract they explain that their stain produces TA's from simple sugars and amino acids. Did they ever show that amino acids are essential? Which amino acids are essential? Do they mean glutamate and phenylalanine? Did they ever consider increasing or decreasing the amount of these amino acids in the medium to see what effects it had (if any) on TA production?

12. Is Supplementary Fig. 5 missreferenced in line 148? It is referenced when mentioning the integration of codon optimized AbCYP80F1 and DsH6H, but the figure shows the screening of different H6H orthologs.

13. In line 249, they report the production of ~ 1 ug/L and ~0.1 ug/L of hyoscyamine and scopolamine, but it is not clear what intermediate was fed, if any. If none, then what is the difference between these experiments and those done with the strain described a few lines below CSY1296, which exhibited

production of these compounds de novo, which presumably means from glucose. This needs to be clarified.
14. What is the difference between disruption and deletion in line 300?

Comments

1. The authors propose an enzymatic mechanism for the newly discovered HDH based on structural modeling of its sequence on to the crystal structure of a synapyl alcohol dehydrogenase and ligand docking. While the approach is valid and often used, it is not as rigorous or conclusive as a careful structural/biochemical mechanistic characterization (despite the inactivation results obtained with various mutants they tested – as there are many ways to break an enzyme). Thus it would be appropriate for their language to reflect this caveat, and discuss how this mechanism compares with the mechanism(s) elucidated or proposed for other synapyl alcohol dehydrogenases, as well as consider any biases derived for having used a member of this class of dehydrogenases to build their model in the first place.
2. They need to add the following references:
a. For the recently identified PLA-UGT (Line 79).
b. For the cytochrome P450 littorine mutase (CYP80F1), (Line 145)
c. For AbCYP80F1 (Line 148) and DsH6H (Line 148)
3. While it is not the first example, it's still not every day that one reads about a complex biosynthetic pathway that involves so many different subcellular compartments. Thus, the authors miss the opportunity to discuss how each of them contribute to the biosynthesis of the final products. It would be great if they briefly discussed each of their essential roles: why is DmMPO1 targeted to the peroxisome? Why it's better to keep Arg2p in mitochondria as opposed to moving Arg biosynthesis to the cytosol? What is the special contribution of the ER/Golgi for tropine and hyoscyamine biosynthesis? And especially, why is AbLS only active in the vacuole? While they may not have answers to all these questions, I think readers would appreciate the authors' rationales and hypotheses.

Referee #3 (Remarks to the Author):

This work demonstrates the complete biosynthesis of hyoscyamine and scopolamine from simple, standard feedstocks in yeast. It relies on modular pathway engineering requiring a complex interplay between chromosomal modifications, heterologous expression, compartmentalization, and transport. Srinivasan et al. identified HDH as performing the key missing reaction to convert hyoscyamine aldehyde to hyoscyamine allowing for the ultimate production of scopolamine. The work is an impressive piece of engineering appropriate for the journal as well as having real world significance relating to supply-chain and resource availability issues surrounding sourcing tropane alkaloids from plants.

My one comment is because the titers are currently low, if there could be more discussion on the confidence and precedence of increasing the titers to industrially relevant levels with such a complex and presumably metabolically burdensome pathway.

Author Rebuttals to Initial Comments:

Response to Reviewer Comments

We would like to thank all three reviewers for their helpful comments and suggestions for improving our manuscript. In the sections below, we address each comment made by the reviewers. In addressing several reviewer questions and comments, we have expanded on and added additional Supplementary

Notes due to manuscript length restrictions.

Reviewer #1 (Remarks to the Authors):

> *The paper is well written and excellent in design and performance of the experiments and data interpretation. I do only have minor comments as you can see in the attached file. Besides all the interesting genetic and metabolic engineering work, we must be aware that a full structural elucidation of scopolamine and hyoscyamine is not possible, because no information on chiral stereoisomers is given. It would be interesting to know, if the authors have considered chiral activity and if the amount of all products is sufficient to carry out this experiment.*

We thank the reviewer for their supportive remarks, and their suggestions for strengthening the Introduction text. We agree that consideration of product stereochemistry is important for reporting on the natural products from our microbial biosynthesis platform. We did not use chiral separation methods for detecting or quantifying the TA products, as the titers from the reported strains would have necessitated prohibitively large-scale purifications for chiral chromatography or NMR workflows. However, as detailed below, we did consider the stereochemistry inherent to the enzymes used in our pathway when designing experiments and reporting which products were synthesized by our engineered strains.

It has been previously demonstrated that the enzymes in Module IV of the pathway are highly stereospecific. Littorine mutase (CYP80F1) has been shown to only catalyze the rearrangement reaction of (R)-littorine to (S)-hyoscyamine aldehyde; if presented with (S)-littorine, a range of hydroxylated intermediates are synthesized instead of the aldehyde[1,2]. Similarly, prior structural and kinetic characterization of the H6H enzyme has demonstrated that it catalyzes the two-step hydroxylation and epoxidation of (S)-hyoscyamine to (S)-scopolamine only, and shows negligible activity on (R)-hyoscyamine[3]. The remaining uncertainty regarding stereochemistry pertains to biosynthesis of littorine, as the stereospecificity of littorine synthase was not tested or discussed in the paper in which it was initially reported[4]. The stereochemistry of littorine is dictated by that of phenyllactic acid (PLA), with D-PLA (i.e., (R)-PLA) leading to production of (R)-littorine. The stereochemistry of PLA is, in turn, set by the stereospecificity of the phenylpyruvate reductase (PPR), which in our strains was *Wickerhamia fluorescens* PPR—previously demonstrated to be a D-specific PPR[5]. As neither the UDP-glucosyltransferase, the littorine synthase, nor the newly reported hyoscyamine dehydrogenase (HDH) reactions act on the chiral center of the TA intermediate or involve an achiral intermediate, no inversion of stereochemistry occurs in these reactions.

Based on the above biochemical logic and the previously established stereospecificities of the enzymes in Modules III and IV, we can deduce that only (R)-littorine, (S)-hyoscyamine, and (S)-scopolamine were produced *de novo* by our engineered strains. Additionally, we ensured that specific stereoisomers were used for substrate feeding experiments: (R)-littorine for screening HDH candidates (Fig. 2b, d) and mutants/orthologs (Extended Data Figs. 4, 5), and (S)-hyoscyamine for screening H6H orthologs (Extended Data Fig. 3). Therefore, we can additionally conclude that these same TA stereoisomers were produced in feeding experiments. However, we agree with the reviewer that this sequence of biochemical logic to establish stereochemistry is not obvious, and have therefore included an additional Supplementary Note 9 to clarify this point.

Reviewer #2 (Remarks to the Authors):

This manuscript reports a complete biosynthetic pathway for the conversion of glucose (and amino acids) to hyoscyamine and scopolamine, two FDA approved drugs belonging to the tropane alkaloids' family of natural products, which includes several other valuable pharmaceuticals (atropine) and stimulants (cocaine). This study is a tour de force that integrates many previously known elements (the biosynthesis of different intermediate metabolites, involvement of subcellular organelles, and the final enzyme for scopolamine production) with new discoveries and technical developments that enabled this achievement. These include functional genomics to discover the hyoscyamine dehydrogenase essential to complete the pathway, identification of functional enzymes to produce PLA-glucoside (AbUGT) and littorine (DsRed-AbLS) in yeast, a novel synthetic strategy to traffic heterologous enzymes across the ER/Golgi and into the vacuole, and the demonstration that heterologous intracellular transporters (from plant) can be functional in yeast. I have no major concerns with the execution and overall conclusions of this study, and believe it is an important contribution to the advancement of plant natural product synthesis in microbes, but there are some important questions and comments that the authors need to address before it is ready for publication.

We thank the reviewer for their insightful remarks, which we feel have enabled us to improve the clarity and impact of our manuscript. We address each of the reviewer's questions and comments on a point-by-point basis below.

Questions:

1. *The authors mention there is a putative N-terminal signal peptide in AbLS that targets the enzyme for trafficking across the ER to the vacuole in plants (Line 211). So it is confusing that fusing a GFP on the N-terminus of this enzyme would still result in it being targeted to the vacuole. Wouldn't this N-terminal fusion disrupt the functionality of the signal peptide? Does this imply that the signal does not need to be in the N-terminus? Or does it mean it is in fact not involved in this trafficking? Could it be that other internal sequence they later uncover? They need to clarify what is going on here.*

We thank the reviewer for pointing out the potentially confusing description of the role of the signal peptide (SP) in AbLS trafficking. As stated on line 211 of the original manuscript, the putative N-terminal SP is expected to enable AbLS trafficking to the vacuole in plants. However, this is not necessarily the case in yeast: it is possible that the vacuole-targeting sequence elements in the SP are plant-specific, and were not recognized by the yeast trafficking machinery when AbLS was heterologously expressed in our engineered strains. This is consistent with our observation that AbLS (with an unmodified N-terminus) fails to pass the yeast trans-Golgi network (Extended Data Fig. 7e, f), and supports our hypothesis that wild-type AbLS does not successfully reach the yeast vacuole. As the SP presumably enables vacuole targeting in plants, but fails to do so in yeast, we co-opted the SP to develop a novel engineering strategy that allows AbLS to reach the yeast vacuole using an alternative route.

In yeast, proteins can follow two different trafficking routes from the ER via the Golgi to the vacuole: soluble proteins which reach the Golgi require a vacuole-targeting sequence for egress to the vacuole, whereas transmembrane proteins which reach the Golgi appear to be routed to the vacuole by default[6,7]. We hypothesized that masking the SP of AbLS using a fused N-terminal domain (such as GFP or DsRed) converts soluble AbLS into a transmembrane protein where the SP serves as the transmembrane anchor, enabling the engineered enzyme to reach the yeast vacuole without a specific vacuole targeting sequence. The microscopy in Fig. 3b supports this hypothesis, showing that GFP fused to the N-terminus of AbLS—now with a medial SP/transmembrane domain—enables localization to the yeast vacuole. N-terminal fusion does not necessarily disrupt SP activity, as naturally-occurring single-pass transmembrane proteins rely on internal signal peptides for localization to and orientation in the ER[8]. In the context of our engineered fusion enzyme, the hypothesized role of the SP is twofold: (i) to direct the nascent polypeptide to the ER, where it can enter the secretion pathway (a role that is retained from

the original N-terminal SP, and for which the SP need not be located at the N-terminus); and (ii) to act as a transmembrane anchor that enables the entire transmembrane fusion protein to be trafficked from the Golgi to the vacuole without needing a vacuole-targeting sequence. We suspect that masking the N-terminus of the SP inhibits SP cleavage and removal in the ER, such that it remains as a transmembrane domain within the fusion protein as it is trafficked to the vacuole (see response to Questions 9 and 10 for updated microscopy analysis supporting this). The other internal propeptide that was later detected (about ~2/3 through the AbLS domain) shows no signal sequences or transmembrane character based on *in silico* prediction software, is a highly variable region in plant SCPL-ATs, and is removed during protein maturation for some SCPL-ATs (e.g., *Arabidopsis* SCT). Thus, it is unlikely to contain signal elements important for enzyme localization.

To summarize:

- The SP likely enables AbLS to be trafficked to the vacuole in plants;
- The SP alone fails to enable AbLS trafficking to the vacuole in yeast, necessitating an alternate mechanism for vacuolar localization;
- Converting the N-terminal SP into an internal transmembrane domain within a fluorophore-AbLS fusion protein may enable the protein to be trafficked from the Golgi to the yeast vacuole without needing a yeast-specific vacuole targeting sequence.

It should be noted that we make no claims about the effect of this N-terminal fluorophore fusion on AbLS localization in plants; it is possible that this strategy actually disrupts the canonical vacuolar trafficking *in planta*. We only suggest that the fusion strategy provides an alternate route for AbLS to reach the vacuole in yeast, since any native signal sequences in AbLS appear insufficient to achieve this targeting in yeast. To clarify the hypothesized role for the signal peptide in engineered AbLS expression and function, we have included the above discussion in Supplementary Note 8.

2. *Similarly, they mention in line 216 that the C-terminus of this enzyme is essential for stability, but they later show that they can detect enzymes with the HA-peptide fused to the C-terminus (Figs.S10, S11, and S13). They need to clarify this apparent contradiction.*

We thank the reviewer for pointing out this contradictory language. The study that is cited on line 216 of the original manuscript observed that an unmodified C-terminus was essential for retaining the enzymatic activity of the SCPL acyltransferase SMT from *Arabidopsis* when secreted by yeast and purified from culture media. This suggests that an unmodified C-terminus may be crucial for SCPL-ATs to fold into an active conformation—but may not be crucial for gene expression and polypeptide translation. It is therefore possible that in our experiments, AbLS modified with a C-terminal HA tag is still expressed and translated but not correctly folded, and was therefore still detectable by Western blot. This is consistent with our failure to observe any fluorescence from AbLS with C-terminally fused GFP—an unfolded AbLS domain may have impeded folding of the C-terminal fluorophore. However, we agree with the reviewer that our use of the term ‘stability’ is ambiguous. Therefore, we have clarified the language in the revised manuscript used in citing that reference, replacing ‘stability’ with ‘folding’.

3. *The authors found that AbLS is N-glycosylated in yeast and in tobacco, and using site-directed mutagenesis identified the glycosylation sites and determined they are the same in both yeast and tobacco. However, AbLS is heterologous in both systems so these results do not necessarily reflect what goes on inside A. belladonna. What are the glycosylation similarities between tobacco and A. belladonna? Are they assuming they are the same? Beyond the specific glycosylation sites, are the glycosylation moieties the same between both plants and between them and yeast? It seems appropriate for the authors to expand their discussion/explanations on these points (or add another supplementary note if they are lacking space). Additionally, point mutations in yeast cause incomplete or lack of glycosylation for a fraction of the enzyme (e.g. lane 5 Fig 11B) but not in tobacco (e.g. lane 7 Fig S11A). There are also clearly observable heavier (~150KDa) and lighter (~27-37KDa) bands*

in enzymes expressed in tobacco but not in yeast, suggesting there are fundamental differences in glycosylation and processing that are not discussed.

We thank the reviewer for raising these points regarding AbLS N-glycosylation patterns. We had originally limited our discussion of these patterns to determine whether differences in N-glycosylation between tobacco and yeast might be the primary driver of the lack of AbLS activity in yeast. However, we agree that a more comprehensive discussion of these glycosylation patterns may be important for understanding the processing requirements for AbLS activity in different biological contexts. Therefore, we have expanded Supplementary Note 6 to provide a more complete discussion of these glycosylation patterns, summarized below.

The reviewer's point regarding glycosylation differences between different plants, and between plants and yeast, is a valid concern. Prior studies have established that plants, yeast, insects, and mammals all share a highly conserved set of ancient protein N-glycosylation machinery, and as a result also share a conserved core glycan structure[9]. N-glycosylation patterns have also been found to be well-conserved across higher plants, and particularly across plants of the same family[9,10]; based on this similarity, tobacco has been previously used to study N-glycosylation of heterologous proteins from other *Solanaceae* like tomato[11,12]. Moreover, the prior study which reported the discovery of AbLS also observed that the enzyme retains its activity when expressed in tobacco[4]. Based on these points, we assumed that N-glycosylation patterns (both loci and moieties) between *A. belladonna* and tobacco must be sufficiently similar to enable correct folding, processing, and activity in the latter, justifying its use as a substitute for the former to compare N-glycosylation patterns to those of yeast.

The reviewer is justified in questioning whether N-glycosylation moieties are conserved between *Solanaceae* (or higher plants in general) and yeast. Yeast proteins share the same core glycan structure as those of plants but tend to be more heavily mannosylated, typically existing across a spectrum of mannosylation levels[9]. Based on this, and the observed overlap of yeast- and tobacco-expressed AbLS bands by Western blot (Extended Data Fig. 7b, c), we concluded that at least some fraction of the yeast-expressed AbLS was in a similar glycosylation state to the tobacco-expressed AbLS. By "similar state", we meant that the same sites were occupied by glycosylation moieties comparable in structure and size (i.e., same degree of mannosylation). However, the precision of N-glycosylation structure required for activity of any particular plant enzyme is unclear; for example, once a site has at least 10 mannose residues, does adding extra mannose residues (as might occur during heterologous expression in yeast) cause problems? Further characterization of these precise N-glycosylation differences and requirements would be a useful avenue of further study, but we feel is beyond the scope of our study.

As the reviewer points out, the Western blot results in Extended Data Fig. 7 do indicate some intriguing differences in AbLS glycosylation patterns between tobacco and yeast, such as incomplete vs. complete glycosylation of point mutants and the presence of additional high (~150 kDa) and low (<30 kDa) molecular weight bands in tobacco. We point out that the additional high-MW bands are also visible in the yeast protein samples, but are much fainter (Extended Data Fig. 7b, c, f). Although we cannot conclusively identify the source of these additional bands or the glycosylation differences between point mutants without extensive additional experiments which we feel are outside the scope of our study, we discuss our hypotheses for these observations in the amended Supplementary Note 6. Overall, we feel that these observations do not invalidate our major conclusions from the N-glycosylation experiments: we are not claiming that glycosylation differences in yeast and plants are not relevant to LS activity; rather, we are claiming that these differences are not likely to be the *primary* factor limiting LS activity in yeast (i.e., there is likely some other underlying primary failure mode), and that further characterization of glycosylation patterns was therefore not a priority for AbLS troubleshooting.

4. *Related to 3, are the glycosylation sites important for expression, and more importantly for activity of AbLS in yeast? Did they ever test their quadruple mutant (or single mutants) for activity (in the cell)?*

We thank the reviewer for raising this point regarding the role of glycosylation in AbLS activity. We can deduce that the glycosylation sites are not essential for expression in either yeast or tobacco, as we were able to see strong expression of both wild-type and glycosylation mutants (including the quadruple mutant) by Western blot (Extended Data Fig. 7b, c). We did not test any of the glycosylation point mutants for activity in either yeast or tobacco. However, given that wild-type AbLS was functional in tobacco but non-functional in yeast, while DsRed-AbLS (with identical, unmodified N-glycosylation sites) was functional in yeast, we can at least infer that N-glycosylation was not a primary limitation of AbLS activity. We acknowledge that further characterization of the functional role of the N-glycosylation sites may be useful for improving the heterologous activity of this enzyme, but also feel that the requisite mutagenesis and yeast/plant testing experiments are beyond the scope, length, and time constraints of our manuscript. We have, however, included a brief discussion of these experiments as avenues of future work in the expanded Supplementary Note 6.

5. *In line 159, the authors suggest that the internal SP of AbLS blocking its active site explains why the enzyme is inactive in yeast (including in different compartments). However, when they fused this enzyme to soluble fluorophores (to resolve putative TGN sorting obstruction) the problem seemed to go away. How can fusion of these fluorophores prevent the internal pro-peptide from blocking the active site? Related to question 1, how is an N-terminal fusion meant to disrupt TGN targeting still end up targeting the enzyme to the vacuole? Figure 4B indicates the enzyme still makes its way through the Golgi and into the vacuole. It is also not clear (or easy to find) what is the origin of the transmembrane domain in the engineered enzyme. Again, what happened to the pro-peptide in the final, successful, construct? I think the authors need to give a better explanation of their enzyme engineering description and rationale to clarify these questions, especially given that this is such an important part of the story (novelty and results).*

We thank the reviewer for raising these questions regarding the relationship between the putative internal propeptide and AbLS maturation and trafficking. In the text around line 159 of the original manuscript, we had initially hypothesized that the internal propeptide might block the AbLS active site based on homology modeling studies, and that its failure to be removed may be responsible for the lack of activity in yeast. This hypothesis motivated us to examine the processing of AbLS split and propeptide variants by Western blot (Extended Data Fig. 7e, f). However, the predicted differences in protein processing were not supported by the Western blot experiments, which showed that AbLS does not undergo proteolytic propeptide removal in either yeast or tobacco (where it is known to be functional[4]), nor by activity screens of the split and propeptide variants in yeast—leading us to reject this hypothesis and continue searching for other failure modes. Therefore, we believe it is unlikely that the resolution of AbLS activity upon fusing N-terminal fluorophores is related to the internal propeptide sequence. To improve the clarity and coherence of the relevant results section, we have moved the discussion of the role of the propeptide sequence to Supplementary Note 7.

Regarding the reviewer's question about the effect of N-terminal fusion on protein sorting from the TGN, we refer to our response to Question 1. We hypothesized that N-terminal fusion of a soluble fluorophore domain converts the fused AbLS into a transmembrane protein, which is sorted to the vacuole via the Golgi by default in yeast[6,7]. In this context, the transmembrane domain in the engineered enzyme *is* the signal peptide—its sequence is strongly predicted to be a transmembrane domain due to arrangement of highly hydrophobic residues (and this is consistent with the function of SPs in some single-pass transmembrane proteins). This modification does not necessarily bypass the Golgi compartment as the reviewer seems to be suggesting, nor does it 'disrupt' TGN targeting; rather, it shifts the protein from the 'stalled' soluble-protein trafficking pathway (which is where we suspect wild-type AbLS becomes stuck) into a parallel transmembrane-protein trafficking pathway (which is still dependent on the Golgi, but whose default destination is the vacuole).

The reviewer's last point is valid, as we did not verify the fate of the internal propeptide within AbLS in the functional DsRed-AbLS construct. It is therefore possible that our N-terminal fluorophore

fusion strategy affects whether or not the propeptide is processed or removed. However, given that wild-type AbLS is functional in tobacco[4] without propeptide removal (as we showed via Western blot in Extended Data Fig. 7), we can conclude that propeptide removal is not required for activity, and therefore likely does not occur for DsRed-AbLS in yeast either.

To further clarify the hypotheses and rationales involved in the development of our protein engineering approach, as well as to address the remaining uncertainty around the role and fate of the internal propeptide and potential strategies for further structural characterization, we have included these points in an additional Supplementary Note 8.

6. *Related to question 5, the authors attribute the effect of the different fluorophores on the activity of AbLS to their oligomeric states, with higher oligomeric states leading to higher activity. Why is oligomerization important for activity? What GFP version did they use (GFP naturally makes dimers, not monomers)?*

We thank the reviewer for pointing out the ambiguity around the effect of enzyme oligomerization and the specific fluorophores used. The mechanism by which N-terminal domain oligomerization affects the activity of the C-terminal AbLS domain—if indeed it does—is not clear at this time. We suspect that oligomerization may inhibit cleavage and removal of the signal peptide, thereby promoting its retention as a transmembrane domain needed for trafficking from the Golgi to the yeast vacuole. As some serine carboxypeptidases are known to homo-dimerize or -oligomerize[13], it is additionally possible that certain SCPL acyltransferases such as AbLS also require oligomerization for activity. As the structural characterization experiments necessary to test these hypotheses are well beyond the scope of our engineering study, we have addressed these hypotheses in Supplementary Note 8.

The reviewer is justified in raising the question of which specific fluorophore variants were used, as different mutants of the same fluorescent protein are known to exhibit different oligomerization tendencies. In our fusion experiments, we used yEGFP, ytagBFP, ymVenus, ymCherry, and yDsRed.T3 (where 'y' indicates sequence optimization for yeast expression). As the reviewer notes, EGFP does have a very weak tendency to form transient dimers at high concentrations—more so than tagBFP and mVenus, but less so than *Discosoma*-family RFPs such as mCherry or DsRed. To more accurately reflect this oligomerization hierarchy, we have rearranged the order in which the N-terminal FP domains are shown in Fig. 3c (originally 3D), from strictly monomeric (tagBFP, mVenus, SUMO*) to monomeric / weakly dimeric (EGFP, mCherry) to strongly homodimeric (AbUGT) to strongly homooligomeric (DsRed.T3). However, we note that the general correlation between AbLS activity and N-terminal domain oligomerization still appears to hold true. We have also specified in the text the specific fluorescent protein variants used in these experiments.

7. *Also not clear why the presence of a degradation product ~37KDa in Fig S13 leads the authors to conclude that the protein signal in AbLS is not specific to this enzyme. Couldn't it be that tobacco is able to recognize it as a peptide that needs to be processed even if it's specific to AbLS? Perhaps the term "specific" is not clear.*

We thank the reviewer for pointing out the uncertainty regarding the origin of the 37 kDa band. The relevant text in Supplementary Note 8 and the caption of Supplementary Fig. 13 of the original manuscript was meant to present our deduction that this 37 kDa band was not a product of AbLS proteolytic processing. However, we acknowledge that the original text describing this band was ambiguous, and have clarified as described below.

a. If AbLS is indeed processed via propeptide removal (as many other SCPL-ATs are; see Extended Data Fig. 8a), the resultant N- and C-terminal subunits would be ~33 kDa (37 kDa with HA tag) and ~18 kDa (22 kDa with HA tag), respectively. As we used C-terminal HA-tagged AbLS for immunoblotting, we should have observed the ~22 kDa smaller subunit rather than the larger N-terminal 37 kDa fragment, assuming AbLS was indeed processed *in planta*. Our quadruple point

mutant experiments in Extended Data Fig. 7b,c indicate that the total mass of all AbLS N-glycosylations is ~6-8 kDa, meaning that N-glycosylation cannot account for this ~15 kDa discrepancy. This suggests that the 37 kDa band is not a product of AbLS proteolytic processing.

b. We observed the 37 kDa band in samples containing both N- and C-terminal HA-tagged AbLS, as well as AbLS with no HA tag at all (Extended Data Fig. 7d). The observation of identical fragment sizes regardless of which terminus was HA-tagged is inconsistent with the expected N- and C-terminal subunits. Our observation of the 37 kDa band in the non-HA-tagged sample (Extended Data Fig. 7d, lane 1) suggests that this band may represent an AbLS degradation product that is non-specifically recognized by anti-HA antibodies, rather than a product of normal protein processing.
c. In our AbLS deglycosylation experiment (Extended Data Fig. 7a), we observed that the size of the 37 kDa band did not change even as the full-length ~55 kDa AbLS band in the same lane was reduced in apparent molecular weight by N-deglycosylation (lane 2). If the 37 kDa band was indeed a product of AbLS processing, then the deglycosylation of N152 (contained in the predicted 37 kDa subunit) should have produced a visible downward shift on the blot—which we did not observe.

Collectively, these observations and lines of reasoning led us to conclude that the observed 37 kDa band could not have been a product of AbLS proteolytic processing. However, the reviewer is justified in pointing out that the terminology we used to present this conclusion (i.e., usage of 'nonspecific band') was unclear. Therefore, we have amended the relevant text in Supplementary Note 7 to include the more detailed rationale for the origin of the 37 kDa band which we have presented above.

8. *How come a 37KDa band is observed in Figs. S10 and S13, but in Fig. S11 there is a ~30KDa band instead? What is the difference between these experiments to account for this?*

We thank the reviewer for bringing this unexplained variation to our attention. As stated in our response to Question 7 above, our observations and reasoning led us to conclude that the 37 kDa band is likely a result of AbLS degradation—during expression *in vivo*, sample preparation, electrophoresis, or some combination thereof. The reviewer is not entirely correct in stating that a 30 kDa band has replaced the 37 kDa band in Extended Data Fig. 7b,c; the 37 kDa band is still present (see, for example, lane 4 in panel b), only much fainter and not visible in some lanes relative to panels a and d. The additional lower-MW (~25-30 kDa) bands which we believe the reviewer is referencing may also be degradation products of different glycosylation site mutants. The variation in the intensity of these lower-MW (~20-37 kDa) bands also suggests that they may be products of AbLS protein degradation during sample preparation and processing: for example, we did notice some variation in the mechanical properties of the tobacco extracts (viscosity, fraction of insoluble matter, etc.) which may have caused variations in protein exposure to heat and chemical denaturation. These differences, combined with small variations in gel-to-blot transfer efficiency for low-MW proteins, may have produced the observed differences in the size and abundance of different degradation products between samples and blots. However, as such variations did not appear to affect the size or abundance of the primary AbLS bands in any of the blots, we feel that our primary conclusions regarding AbLS N-glycosylation and processing are still supported by the data. To address the variation in the 37 kDa (and other low-MW) bands, we have included the above clarification in Supplementary Notes 6-7.

9. *In Fig S14, DsRed-AbLS looks like it's located in the lumen of the vacuole rather than the vacuolar membrane, yet the authors conclude it is anchored to the membrane with the catalytic domain inside the lumen. What evidence do they have that this is the correct topology of the enzyme? If it's truly on the membrane, why doesn't it look more like NtJAT1-GFP (same figure) or FM4-64 in Fig 3B?*

We thank the reviewer for pointing out the ambiguity in the apparent localization of DsRed-AbLS. We agree that a comparison of DsRed-AbLS and NtJAT1-GFP localization in Supplementary Fig. 14 of the original manuscript may appear inconsistent with both proteins localizing to the vacuolar membrane, as claimed. As we have previously observed increased bleed-through of out-of-focus signal in the red

channel relative to the green channel of the epifluorescence microscope setup, we suspected that this bleed-through effect may have artificially increased the red fluorescent signal observed in the lumen of vacuoles. To eliminate this effect, we performed 2D digital deconvolution analysis, a common computational technique used for removing out-of-focus light distortion from 2D images of 3D structures[14], on both the FM4-64/DsRed and GFP channels of the original images in Fig. 3b and Supplementary Fig. 14. We believe the resultant deconvolved images (Extended Data Fig. 9 of revised manuscript) now provide strong evidence for the vacuolar membrane localization of both DsRed-AbLS and NtJAT1-GFP.

Our conclusion that DsRed-AbLS is located on the vacuolar membrane with the catalytic domain in the lumen is based on both the microscopy data and the topological constraints inherent to inter-compartmental trafficking pathways. As our Western blot data show that wild-type AbLS becomes glycosylated in yeast (as well as in plants), we can infer that the wild-type protein must be oriented facing the lumen of the Golgi, so that it is accessible to glycosylation machinery. Membrane topology is conserved throughout a trafficking pathway, such that any protein domain in the lumen of an organelle (ER, Golgi, vacuole, etc.)—topologically equivalent to the extracellular medium—is retained as such. As N-terminal fusion of a soluble domain (e.g., DsRed) does not change the polarity of the signal peptide, which determines topology of insertion into the ER membrane during translation[15], we can deduce that DsRed-AbLS must also be expressed such that the catalytic AbLS domain remains in the lumen of the ER, Golgi, and ultimately the vacuole during trafficking.

10. *Also in Fig S14, it is difficult to observe localization of NtMATE2-GFP on the PM or vacuolar membrane. It looks like most of the protein is localized to the lumen of the vacuole or localized throughout the cytosol (given it's a membrane protein, this could be explained by a partially proteolyzed protein leaving soluble GFP in the cytosol). Can the authors provide additional evidence that this protein is indeed targeted to the membranes they are claiming (PM and vacuole)? Otherwise, can they make a stronger argument for why these images show what they claim they show? Otherwise they should revise their claims summarized in Fig 4B, or at least be more cautious with their language to reflect the unconvincing evidence they show for this claim in Fig S14.*

We thank the reviewer for pointing out the ambiguity of NtMATE2-GFP localization based on the microscopy images. As described in our response to Question 9 above, we also performed 2D digital deconvolution analysis of the NtMATE2-GFP and DsRed-AbLS images from panel B in Supplementary Fig. 14 of the original manuscript, and have included the results in Extended Data Fig. 9 of the revised manuscript. The deconvolved images clearly show that the bulk of NtMATE2-GFP appears to localize to the vacuolar membrane, with a smaller fraction localizing to the plasma membrane to produce a faint ring of green fluorescence around the perimeter of the cell. We believe the deconvolved images in Extended Data Fig. 9 now provide strong evidence to support our conclusions regarding localization of DsRed-AbLS, NtJAT1-GFP, and NtMATE2-GFP. The methodology for the deconvolution analysis is included in the Methods of the revised manuscript.

11. *In the abstract they explain that their strain produces TA's from simple sugars and amino acids. Did they ever show that amino acids are essential? Which amino acids are essential? Do they mean glutamate and phenylalanine? Did they ever consider increasing or decreasing the amount of these amino acids in the medium to see what effects it had (if any) on TA production?*

We thank the reviewer for raising the issue of amino acid optimization in the growth medium. We only tested strains grown in media with supplemented amino acids and standard nutrient drop-out formulations, and did not determine which amino acids in the media (if any) were essential for TA production in our strains. The only specific amino acid optimization experiments we performed pertained to the role of leucine auxotrophy vs. prototrophy on conversion of hyoscyamine to scopolamine, which were mentioned in the text. While we concur with the reviewer's suggestion that amino acid optimization

and supplementation experiments (e.g., glutamate to improve the arginine branch / tropine, phenylalanine to improve the phenylpropanoid branch / PLA glucoside) would be useful, we feel that these media optimization studies fall under the category of fermentation optimization, and are therefore beyond the scope of this initial proof-of-concept paper. In addition, based on our previous observations of TA titers produced by strains with different auxotrophies, we found that leucine enrichment may also be useful for improving TA production. However, unlike the suggested glutamate and phenylalanine feeding, this strategy was not obvious based on examination of pathway precursors. As such, we feel that amino acid optimization as a general strategy to improve TA production would also likely be non-obvious, and require substantial additional optimization efforts beyond the scope of this proof-of-concept paper. Nonetheless, we have highlighted the potential utility of amino acid / nutrient optimization experiments as a future strategy for bioprocess optimization in Supplementary Note 12.

12. *Is Supplementary Fig. 5 missreferenced in line 148? It is referenced when mentioning the integration of codon optimized AbCYP80F1 and DsH6H, but the figure shows the screening of different H6H orthologs.*

We thank the reviewer for bringing this potentially confusing figure reference to our attention. Supplementary Fig. 5 (now Extended Data Fig. 3) is not misreferenced, but the reviewer is correct in pointing out that the link between the figure and the experiment described in line 148 of the original manuscript is not obvious. We included this figure to show that DsH6H was the most active ortholog in our engineered yeast strain, which is why that ortholog was selected for integration. We have clarified the reference to Extended Data Fig. 3 in the corresponding lines of text.

13. *In line 249, they report the production of ~ 1 ug/L and ~0.1 ug/L of hyoscyamine and scopolamine, but it is not clear what intermediate was fed, if any. If none, then what is the difference between these experiments and those done with the strain described a few lines below CSY1296, which exhibited production of these compounds de novo, which presumably means from glucose. This needs to be clarified.*

We thank the reviewer for bringing this ambiguity in experimental description to our attention. In the original text, we specified that the CSY1296 experiment (lines 254-258) measured *de novo* TA production (i.e., without any fed intermediates), but did not explicitly state that this was also the case for the preceding experiments which tested different AbLS N-terminal fusions (original lines 248-251). In revising this section of the results for brevity and clarity, we have now specified these experimental details to better clarify the experiments.

14. *What is the difference between disruption and deletion in line 300?*

We thank the reviewer for pointing out this ambiguous terminology. As used in our manuscript, disruptions refer to genomic modifications where stop codons are inserted into an open reading frame (ORF), or an out-of-frame expression cassette for a different gene is inserted into an ORF—either of which renders the original gene product non-functional. Deletions refer to genomic modifications intended to deactivate a gene product in which the entire ORF is removed. The vast majority of gene knockouts in our strains were disruptions, although a few were deletions; the choice of disruption vs. deletion depended on several practical factors, including the length of the gene, the genomic locus, the availability of suitable Cas9 guide RNA sites, and whether additional genes were being inserted simultaneously. For the sake of clarity, we have amended all relevant instances in the text to use the term ‘disruption’ exclusively, as in the context of gene product deactivation, deletions can be considered a type of disruption. Details on construction of disruptions are included in the Methods section.

Comments:

1. *The authors propose an enzymatic mechanism for the newly discovered HDH based on structural modeling of its sequence on to the crystal structure of a synapyl alcohol dehydrogenase and ligand docking. While the approach is valid and often used, it is not as rigorous or conclusive as a careful structural/biochemical mechanistic characterization (despite the inactivation results obtained with various mutants they tested – as there are many ways to break an enzyme). Thus it would be appropriate for their language to reflect this caveat, and discuss how this mechanism compares with the mechanism(s) elucidated or proposed for other synapyl alcohol dehydrogenases, as well as consider any biases derived for having used a member of this class of dehydrogenases to build their model in the first place.*

We thank the reviewer for pointing out this limitation of the computational modeling approach. The reviewer is correct in noting the tight scope of our enzyme inactivation assay, as our mutagenesis was limited to only those residues previously identified as being involved in the sinapyl ADH catalytic cycle. We felt that mutagenesis focusing on the catalytic residues was sufficient to show that our newly reported oxidoreductase shares catalytic features with other members of its class, and that a more comprehensive mutagenesis study of the enzyme would be better suited for a future structural and kinetic characterization paper. In light of this, we have expanded the corresponding Supplementary Note 3 to include a comparison of HDH's predicted catalytic mechanism to those of related enzymes; a discussion of some additional features and mutagenesis targets for future structural and kinetics studies; and a discussion of the limitations, biases, and assumptions implicit in our homology-based analysis. Regarding biases, we note that the MDR / sinapyl ADH class was not selected for template-based modeling by human judgment or intuition, but rather based on initial phylogeny and BLAST searches, which indicated that the sinapyl ADH family was the most closely related to our novel ADH.

2. *They need to add the following references:*
    a. *For the recently identified PLA-UGT (Line 79).*
    b. *For the cytochrome P450 littorine mutase (CYP80F1), (Line 145)*
    c. *For AbCYP80F1 (Line 148) and DsH6H (Line 148)*

We thank the reviewer for bringing these missing references to our attention. We have included the supporting reference for (a) in the corresponding line of the revised manuscript. Regarding (b): in revising the text, the sentence on line 145 of the original manuscript was modified such that it no longer specifies the general enzyme CYP80F1; therefore, supporting references for this enzyme have instead been included in the relevant lines of the introduction (main text, paragraph 3) where its activity is summarized. Regarding (c): the *AbCYP80F1* and *DsH6H* orthologs specified on line 148 of the original manuscript were not reported in prior literature, but rather identified from unpublished sequences deposited in the GenBank/UniProt databases, and have no corresponding literature references. We have instead listed database accession numbers for these genes in Supplementary Table 2.

3. *While it is not the first example, it's still not every day that one reads about a complex biosynthetic pathway that involves so many different subcellular compartments. Thus, the authors miss the opportunity to discuss how each of them contribute to the biosynthesis of the final products. It would be great if they briefly discussed each of their essential roles: why is DmMPO1 targeted to the peroxisome? Why it's better to keep Arg2p in mitochondria as opposed to moving Arg biosynthesis to the cytosol? What is the special contribution of the ER/Golgi for tropine and hyoscyamine biosynthesis? And especially, why is AbLS only active in the vacuole? While they may not have answers to all these questions, I think readers would appreciate the authors' rationales and hypotheses.*

We thank the reviewer for this suggestion to better highlight our work. We agree that our incorporation of many different subcellular compartments into a single biosynthesis platform is a major contributor to the

overall impact and novelty of our paper. Therefore, we have included an additional Supplementary Note 10 and associated reference in the Discussion which provides a more explicit discussion of the known and/or hypothesized roles of each subcellular compartment, both in the context of our engineered yeast biosynthesis platform and more broadly in the context of TA biosynthesis.

Reviewer #3 (Remarks to the Authors):

> *This work demonstrates the complete biosynthesis of hyoscyamine and scopolamine from simple, standard feedstocks in yeast. It relies on modular pathway engineering requiring a complex interplay between chromosomal modifications, heterologous expression, compartmentalization, and transport. Srinivasan et al. identified HDH as performing the key missing reaction to convert hyoscyamine aldehyde to hyoscyamine allowing for the ultimate production of scopolamine. The work is an impressive piece of engineering appropriate for the journal as well as having real world significance relating to supply-chain and resource availability issues surrounding sourcing tropane alkaloids from plants.*
>
> *My one comment is because the titers are currently low, if there could be more discussion on the confidence and precedence of increasing the titers to industrially relevant levels with such a complex and presumably metabolically burdensome pathway.*

We thank the reviewer for their supportive comments and their suggestion regarding the feasibility of scale-up operations, as it is a common question for proof-of-concept metabolic engineering studies. As we note in the Discussion, the μg/L titers we report here for hyoscyamine and scopolamine are comparable or better than those reported in prior studies for plant pathways of similar complexity reconstituted in yeast[16–18]. Based on the experiences of our own group and others with translating similarly complex heterologous pathways from lab- to industrial-scale, we also provided a reasonable time estimate in the Discussion for the duration of this scale-up and optimization process. Of note, the actual metabolic burden imposed by pathways of this complexity will not strictly be a function of the number of heterologous enzymes, but is generally related to carbon/nitrogen/ATP requirements and the fraction of the maximum theoretical yield that needs to be obtained for commercial objectives, which generally necessitate metabolic and process modeling and are outside the scope of this study. However, due to the higher price of the products that are synthesized through these complex metabolic pathways, the titer and productivity requirements are typically lower (and associated yield requirements are far lower than the theoretical maximum for the system) than those for cheaper commodity chemicals arising from less complex pathways; which can make these complex pathways less metabolically burdensome in comparison.

We agree with the reviewer that the impact of our proof-of-concept study would be improved by providing more details on the feasibility of the future optimization required. Therefore, we have included an additional Supplementary Note 12 which discusses some specific strategies that are likely to be useful in the scale-up of our engineered pathway, including optimization of media and amino acid formulation (as suggested by Reviewer 2), fermentation process optimization, engineering and/or evolution of key limiting enzymes, rewiring of key endogenous metabolic modules to improve flux through our engineered pathway, and resolution of remaining flux losses from the pathway.

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Reviewer Reports on the First Revision:

Referee #2 (Remarks to the Author):

The authors have addressed all my concerns, so I support publication of this study.