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BRIEF REPORT

Targeted gene knock-in reduces variation between transformants in the mushroom-forming fungus *Schizophyllum commune*

[version 1; peer review: 2 approved with reservations]

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Abstract

Gene integration in mushroom-forming fungi currently occurs by the ectopic integration of a plasmid. The locus of integration is unpredictable and, problematically, this generally results in a high variability in gene expression and phenotypes between the transformants. Here, we developed an approach for targeted gene integration (knock-in) in the basidiomycete *Schizophyllum commune* by replacing a 75-bp non-coding region of the genome with a selection marker and an arbitrary gene of interest using CRISPR-Cas9 ribonucleoproteins. To assess the suitability of our method, we compared targeted integration and ectopic integration of the gene encoding the red fluorescent protein dTomato. Targeted integration resulted in a higher average fluorescence intensity and less variability between the transformants. This method may be applied to any gene construct and may therefore greatly increase the efficiency of functional gene analysis in *S. commune*.

Keywords

Targeted gene integration, gene knock-in, CRISPR-Cas9, fungi, mushrooms, genetic engineering

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Plain language summary

Mushroom-forming fungi are an important sustainable food source. Furthermore, they make important medicinal substances and enzymes for biofuel production. To alter the characteristics of mushrooms, it is interesting to look at the effect of the integration of new genes that produce proteins. However, currently this often does not succeed or results in a sick mushroom, because the gene integrates in a random spot in the genome. Here, we developed a method to very precisely place a gene into a selected area with molecular scissors to reduce the variability of protein production. We test this method by inserting red-fluorescent protein and compare targeted and random integration. With targeted integrations, all obtained strains were very similar in the production of the red-fluorescent protein, while random integrations sometimes resulted in no, or very low production. This method can help us to get a better understanding of how mushrooms work and improve their production.

Introduction

Mushrooms are primarily used as a sustainable food source, but they also produce many pharmacological compounds and carbohydrate-active enzymes (de Mattos-Shipley *et al.*, 2016). *Schizophyllum commune* is a mushroom-forming basidiomycete in the order Agaricales and serves as a model for fruiting body development due to its short life cycle and genetic accessibility (Ohm *et al.*, 2010b). Unlike most mushroom-forming fungi, high transformation efficiency can be achieved in *S. commune* with PEG-mediated transformation of protoplasts (De Jong *et al.*, 2010; Ohm *et al.*, 2010a; Vonk *et al.*, 2019). Additionally, an efficient gene-deletion (knock-out) method is available (Vonk *et al.*, 2019). These methods provide quick and efficient genome editing tools to study the function of genes *S. commune*.

The study of gene function often requires the complementation of gene deletions, over-expression of genes, the expression of recombinant genes, or the heterologous expression of genes encoding fluorescent markers (Ohm *et al.*, 2011; Ohm *et al.*, 2013; Pelkmans *et al.*, 2017; Vonk & Ohm, 2021a). The current gene-integration method relies on random integration of a plasmid that comprises the gene of interest and a selectable marker (Schuren & Wessels, 1994). This method reliably creates transformant strains, but expression of the integrated gene of interest can vary considerably between these strains and some do not show any expression of the integrated gene (Van Peer *et al.*, 2009). This can likely be attributed to variations in copy number, to partial integration of the plasmid into the genome (which may result in an incomplete gene), and to the epigenetic state of the locus of integration. Moreover, some strains show phenotypes that are not caused by the integrated gene but by the interruption of a gene at the integration site. It is often difficult to distinguish between these two, which complicates the study of gene function. Fungal genomes generally have a high gene density. The 38.67 Mbp genome assembly of *S. commune* encodes 16,204 predicted genes, resulting in 419.03 genes per Mbp and a coding content of 52.89% (Ohm *et al.*, 2010b). Including regulatory elements of the genome, such as promoters, enhancers, terminators and non-coding RNA transcripts, this results in a very dense genome where random integrations are likely to affect genetic elements at the site of integration.

This high variation of phenotypes between the transformants complicates the study of gene function, especially since the observed phenotype may not even be caused by the gene of interest. For this reason, generally several transformants are phenotyped in an effort to identify the actual phenotype of the gene integration. Instead, it would be beneficial to predictably target the gene of interest to a predetermined locus, leading to consistent expression dependent only on the selected promotor. This approach has already been applied in other fungal model species, including *Saccharomyces cerevisiae* and *Neurospora crassa* (Matsu-ura *et al.*, 2015; Ronda *et al.*, 2015), but not in any mushroom-forming fungus. Previously, homologous recombination (which is required for targeted integration) was not efficient enough in *S. commune* to make this a feasible strategy. Furthermore, transformation in *S. commune* always requires a selection marker, making the method used in *S. cerevisiae* (which does not require a selection marker) unfeasible. However, we recently developed a CRISPR-Cas9-based method that considerably increases the occurrence of homologous recombination (Vonk *et al.*, 2019). Here, we modified this method to efficiently target a gene of interest and a nourseothricin selection marker to a non-coding locus by homologous recombination. We show that this greatly enhances the reproducibility of integration and gene expression compared with ectopic integrations.

Methods

Culture conditions and strains

S. commune was grown from a small agar inoculum on solid *S. commune* minimal medium (SCMM) supplemented with 1.5% agar at 30°C (Van Peer *et al.*, 2009). All strains are derived from *S. commune* H4–8 (*mat*A43*mat*B41; FGSC 9210) (Ohm *et al.*, 2010b). For targeted integration, a previously published *Δku80* strain in a H4–8 background was used (De Jong *et al.*, 2010). For selection on nourseothricin (Bio-Connect, Netherlands) or phleomycin (Bio-Connect, Netherlands), SCMM was supplemented with 15 µg/mL and 25 µg/mL antibiotic, respectively (Alves *et al.*, 2004).

Plasmid construction

The locus selected for targeted integration was on scaffold 6: 2369122–2369196. The flanks upstream and downstream of this region were amplified with spi_up_fw + spi_up_NcoI_rv and spi_down_fw + spi_down_rv respectively (Table 2), resulting in 1106 bp and 1107 bp fragments. The primer spi_up_NcoI_rv introduced an NcoI site into the 3′ end of the upstream fragment for future cloning. The targeted integration plasmid was constructed as previously described for gene deletion plasmids (Vonk & Ohm, 2021a), resulting in plasmid pPV033 (available from Addgene under plasmid #178359). To construct the *dTomato* targeted integration plasmid, the tubulin promoter and *dTomato* were amplified from pPV040 with primers ptub_dtom_fw + ptub_dTom_rv resulting

in a 1475 fragment (Table 2). The *hom2* terminator was amplified from the *S. commune* H4–8 genome with primers hom2_term_fw and hom2_term_rv, resulting in a 1021 bp fragment (Table 2). Primers ptub_dtom_fw and hom2_term_rv contained 20 bp overhangs with pPV033 at the NcoI site. The tubulin promotor with *dTomato* and *hom2* terminator were integrated in pPV033 digested with NcoI using NEB HiFi DNA Assembly Master Mix (New England Biolabs, MA, USA) resulting in plasmid pPV048.

Transformation of *S. commune*

Ectopic integration was performed as previously described (Van Peer *et al.*, 2009). Targeted integration (knock-in) was performed using a similar approach as previously described for gene deletions (knock-out) (Vonk & Ohm, 2021a). A sgRNA was designed on the 75 bp and produced with the primers spi_sgRNA_fw + spi_sgRNA_rv (Table 2) with the GeneArt Precision sgRNA Synthesis Kit (ThermoFisher Scientific, MA, USA) according to manufacturer's specifications (Vonk *et al.*, 2019). The plasmid pPV048 was linearized with Anza NdeI (ThermoFisher Scientific, MA, USA). The candidate transformants were verified by PCR with two primers (spi_chk_fw + spi_chk_rv, Table 2), binding outside the upstream and downstream flanks site of integration. In the case of successful integration, this results in the insertion of 3780 bp at the 75 bp region inside the selected upstream and downstream flanks. For the check PCR this results in a change of band size from 2473 bp in the wild type to 6177 bp in transformants with a successful targeted integration.

Fluorescent imaging and analysis

The transformants and wild type H4–8 were grown for five days in triplicate at 25°C with a 16-hour light, 8-hour dark cycle. Imaging was done with an Euromex sCMED-20 camera on a Leica MZ16FA with a Leica Planapo 0.63× with fluorescent light from a Leica EL6000 at 1 second exposure and 10× digital gain. Pictures were converted to 8-bit grey-scale and the average fluorescence of pixels with an intensity >14 in a range from 0 to 255 was determined in ImageJ version 1.53c (RRID:SCR_003070) and used as an arbitrary unit of measurement of dTomato fluorescence (AU). The fluorescence intensity was normalized by subtracting the measured autofluorescence of the wild type H4–8. Both the raw and processed fluorescence data are available in the extended data (Vonk & Ohm, 2021).

Results

We identified a suitable locus for targeted integration using the following criteria: the neighboring genes (i) should be convergently transcribed and (ii) should be spaced at least 2 kbp apart, to minimize the odds of disrupting regulatory elements in a promoter. The neighboring genes (iii) should have stable expression during development and (iv) active histone 3 lysine 4 dimethylation (H3K4me2) during development (which indicates that the chromatin structure facilitates transcription), to increase the odds of stable expression solely under control of the selected promoter. Based on these criteria, a 2723-bp area on scaffold 6 from 2367903–2370626 was selected (Figure 1a). This region is flanked by a putative vacuolar transporter chaperone (protein ID Schco3|2627341, 57% amino acid identity match to *S. cerevisiae* VTC4) and a fungal-specific transcription factor (protein ID Schco3|2543274) that both have stable expression at approximately 40 FPKM during development (Almási *et al.*, 2019). Furthermore, we previously determined that this region has high H3K4 dimethylation that does not change during development (Vonk & Ohm, 2021a).

We created a plasmid to facilitate targeted integration, comprising a nourseothricin resistance cassette flanked by 1.1 kbp upstream and downstream homology flanks. Any promoter, gene of interest and terminator can be subcloned into an NcoI restriction site between the upstream flank and nourseothricin resistance cassette. Moreover, a phleomycin resistance cassette is located outside the homology flanks, allowing for counter-selection of transformants that did not originate from a double cross-over. Upon successful integration into the genome, this would result in the deletion of 75 bp at the integration site, replacing it with the nourseothricin resistance cassette and the gene of interest (Figure 1). Homologous recombination during the transformation is further induced by a CRISPR-Cas9 ribonucleoprotein programmed to cut in the integration site.

We determined the efficacy of our method by comparing transformants generated by the traditional ectopic integration approach with transformants generated with our novel targeted

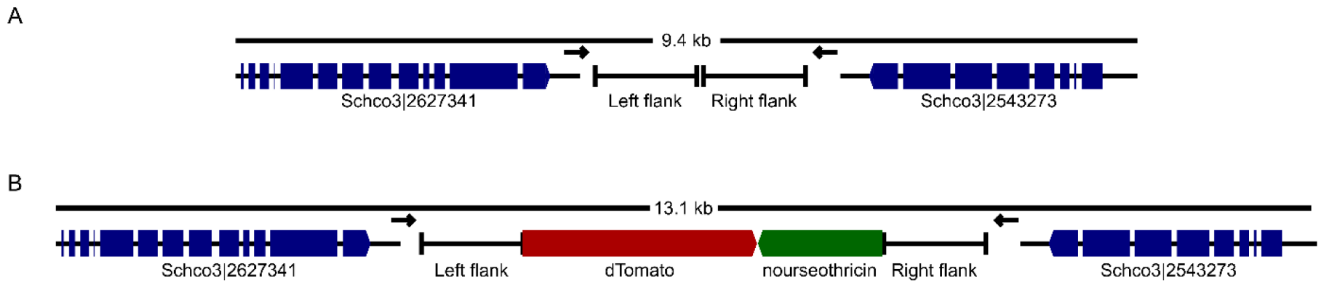

Figure 1. Genomic region of scaffold 6 (2,364,297–2,373,735 bp) before (**A**) and after (**B**) integration of the targeted insertion. Arrows indicate the location of PCR primers to verify successful targeted knock-in transformants.

integration approach. As a marker gene, we used *dTomato* under control of the tubulin promoter (Figure 1b), which allowed us to efficiently determine the variation between the transformants by measuring the colony-wide intensity of red fluorescence as well as its distribution pattern across the colony. We first transformed wild-type *S. commune* with our plasmid but without Cas9-RNPs, which resulted in random integration, as verified by PCR (Figure 2a). 12 nourseothricin-resistant transformants, named E1–E12 were screened for dTomato fluorescence (Figure 2b). The fluorescence intensity varied

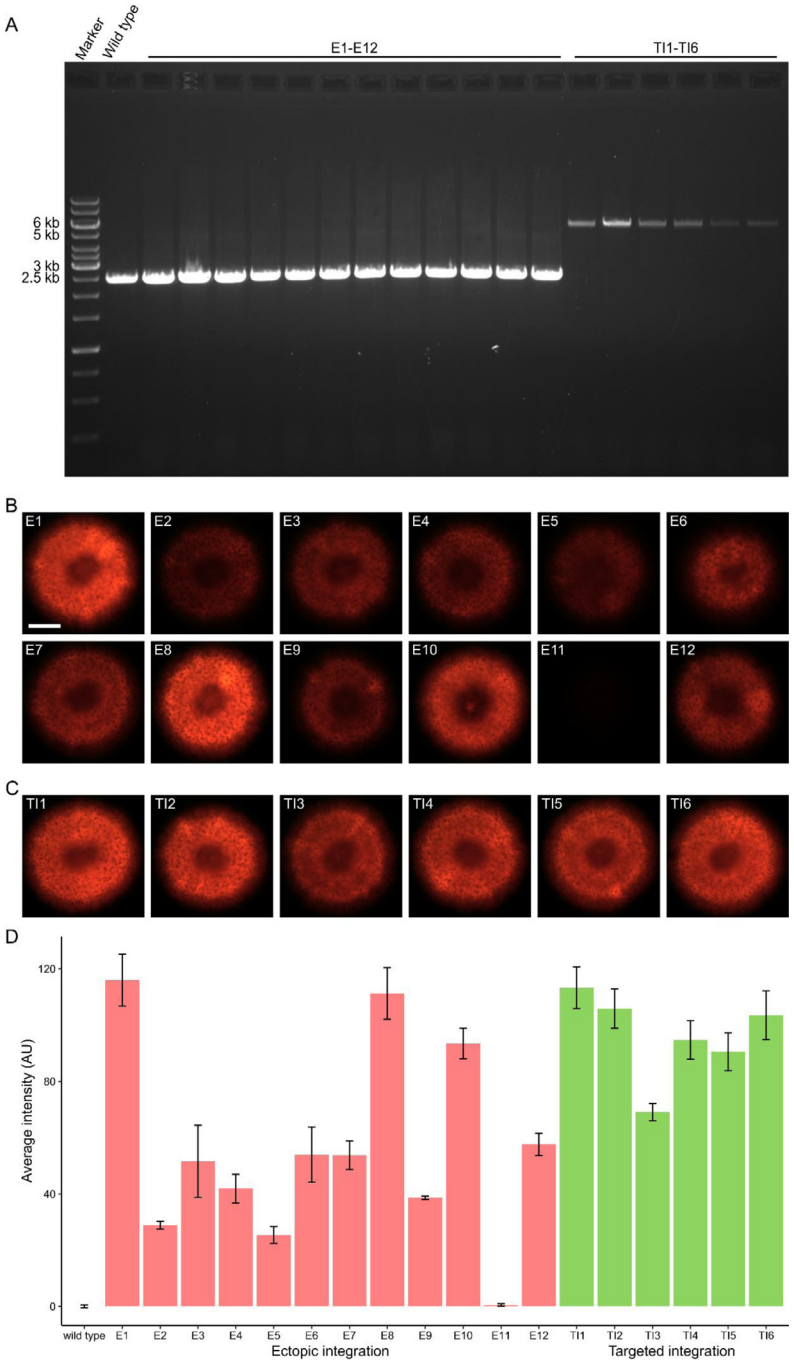

Figure 2. Genotyping and phenotyping the strains transformed with the *dTomato* targeted insertion plasmid. A: PCR on the targeted integration locus. Integration of the *dTomato* expression cassette and nourseothricin cassette results in an increase in band size from 2473 bp to 6178 bp. All ectopic integration strains show the wild type band, while all targeted knock-in strains show the larger band that indicates successful integration. **B**: Strains with ectopic integration of *dTomato*. The white bar indicates 1 cm. **C**: Strains with targeted integration of *dTomato*. **D**: Quantification of fluorescence intensity in arbitrary units. On average, fluorescence is more consistent in strains generated by targeted integration, compared to ectopic integration. The fluorescence was measured in three biological replicates. The raw fluorescence photos and quantifications can be found in the supplementary data.

greatly between the transformants with a spread of 115.4 arbitrary units of fluorescence (AU) between the strain with the lowest and highest fluorescence (0.6 AU and 116 AU, respectively) (Table 1). Strain E11 showed very little fluorescence, while strain E1 had the highest fluorescence (Figure 2d).

Next, we created targeted knock-in transformants by introducing the plasmid in a *Δku80* strain (which is deficient in non-homologous end-joining) assisted by Cas9-RNPs. This resulted in six nourseothricin-resistant, phleomycin-sensitive strains, named TI1–TI6, that were all red fluorescent (Figure 2c). With PCR, it was confirmed that all targeted knock-in transformants integrated the *dTomato* gene and nourseothricin resistance cassette at the targeted locus (Figure 2a). Compared to the ectopic integration transformants, the spread in fluorescence intensity between the lowest and highest fluorescent strain was much lower at 44.2 AU (69.3 AU and 113.2 AU, respectively) (Table 1). Furthermore, the variance was markedly lower. Additionally, the average intensity was 40.1 AU higher in the targeted integration transformants. While no strain showed more intense fluorescence than ectopic integration strain E1 (possibly due to several integrations of the plasmid), only three of the twelve ectopic integration strains had a higher fluorescence than the lowest fluorescence intensity measured in the targeted knock-in transformants (Figure 2d).

Discussion

We developed a method for targeted gene integration (knock-in) in *S. commune*. This method greatly reduced the variation in fluorescence intensity between the transformants from 115.4 AU to 44.2 AU (in transformants resulting from ectopic integration and targeted integration, respectively). Average dTomato fluorescence was also stronger during targeted integration, indicating that the selected region for targeted integration is suitable for expression of genes. Unlike a similar method applied in *S. cerevisiae*, our method does rely on the integration

Table 1. Statistics on dTomato fluorescence after ectopic and targeted integration.

Strain	Average fluorescence intensity	Minimal fluorescence intensity	Maximum fluorescence intensity	Spread	Variance
Ectopic integration	56.1	0.6	116	115.4	1110.96
Targeted integration	96.2	69.1	113.3	44.2	201.2

Table 2. Primers used in this study. Underlined sequences indicate homologous sequences for Gibson assembly.

Primer name	Sequence
spi_up_fw	CTATGACCATGATTACGCCAGCCAACAATCAGATCCGCTAGC
spi_up_NcoI_rv	TCCCAGACCACCATGCCGGGCCATGGAGCAACCTCGGTGCTTATGACT
spi_down_fw	GTCCCCCTCGAGGCGCGCCGGGGTCCTTTCTGACGATGACCA
spi_down_rv	GATAACCTTCAGCAGAACGACGTGACTTTTCGCCGTCTGTAC
ptub_dtom_fw	CATAAGCACCGAGGTTGCTCAAGCTTTGGAACCGGGCCTCAA
ptub_dtom_rv	CTTGTACAGCTCGTCCATGC
hom2_term_fw	GCATGGACGAGCTGTACAAGTAGCCCCTGGCAATCATTTGC
hom2_term_rv	CCCAGACCACCATGCCGGGCTGACCTTGGGGAAAATCAAG
spi_sgRNA_fw	TAATACGACTCACTATAGGTCCTCCAGAGCTCCGCGCCA
spi_sgRNA_rv	TTCTAGCTCTAAAACTGGCGCGGAGCTCTGGAGGA
spi_chk_fw	GCAGATGCTGGATAGGCTTC
spi_chk_rv	GGGACATGGTGGTAGAGAGC

of a selection marker (Ronda *et al.*, 2015). It was previously shown that homologous recombination and transformation efficiency are not sufficient for transformation without a selection marker in *S. commune* and therefore a selection marker is still required (Vonk *et al.*, 2019). Although the transformation efficiency with targeted integration is still markedly lower than with ectopic integrations, it is comparable to the efficiency of Cas9-RNP-mediated gene deletion and therefore sufficient for gene integration. Although the size of the integration was considerably larger (3.8 kbp) than in the case of a gene deletion (1.3 kbp), the transformation efficiency did not appear to be negatively affected.

Together, this method expands on the toolkit available for functional genomics in *S. commune* with reliable and consistent expression of integrated genes. This can be used for the complementation of previously deleted genes, but also the integration of genes modified in their active sites. An example of this is the mutation of the phosphorylation sites of the homeodomain transcription factor *hom2* (Pelkmans *et al.*, 2017). Furthermore, combined with our previously developed ChIP-Seq protocol, it enables efficient integration and expression of epitope-tagged transcription factors to identify their binding sites (Vonk & Ohm, 2021).

Data and software availability

Underlying data

Zenodo: Underlying data for 'Targeted gene knock-in reduces variation between transformants in the mushroom-forming fungus *Schizophyllum commune*'. https://doi.org/10.5281/zenodo.5644994 (Vonk & Ohm, 2021).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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Current Peer Review Status: ? ?

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Reviewer Report 25 July 2022

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Vonk et al establish a strategy for reliable transgene expression in the fungus *Schizophyllum commune* based on CRISPR/Cas9-aided homologous recombination, while testing a new chromosomal target locus for expression. The work builds on their platform for targeted gene deletion in *S. commune* using CRISPR/Cas9 ribonucleoprotein complex transformation (Vok et al 2019).

Achieving reliable heterologous expression is a stepping stone to developing *S. commune* as a standardised chassis for production. The target site at Scaffold 6 is well supported by their in silico analysis of the neighbouring genetic context, and shows experimentally robust fluorescent protein expression.

Regarding the transformation strategy, the differences in expression robustness between targeted integration and random integration are shown and well supported by the literature. However, to fully support that CRISPR/Cas9 RNP is required for efficient integration in the ΔKU background, a control is needed were the template for repair plasmid is transformed alone without CRISPR/Cas9 RNP (to compare to the efficiency of homologous recombination alone).

Considering this is a method report, there are several experimental settings and data regarding transformation efficiency that could be described more precisely to aid future users of this strategy (see comments).

Overall, the manuscript could help the community further develop targeted integration in *S. commune*. The current manuscript is missing some experimental details and controls that could better contextualise and support the proposed strategy. With those additions, the reader would be better set to reproduce the method and build on top of it.

Major comments

1. To fully support that the efficiency of targeted integration is boosted by CRISPR/Cas9 in the

ΔKU strain, a negative control would be needed where the template DNA was transformed without CRISPR/Cas9 RNP.

2. As this is a transformation method, it is important to discuss transformation efficiency. For this, the methods used for transformation should be self-contained in the manuscript and described specifically (instead of referencing previous work). E.g. were the same amounts of template plasmid and protoplasts used for ectopic integration and targeted integration? It is unclear in the current version as in the references Vonk & Ohm, 2021 variable numbers of protoplasts and template were used for different genes; and values also differ in Van Peer et al., 2009. Additionally, it would be interesting to report the transformation efficiency (targeted transformants/ug of donor DNA for a set number of protoplasts).

3. On a similar note, the use of a counterselection strategy is an interesting addition, not always used in CRISPR/Cas applications in other fungi. However, it does add more experimental steps (and time). Could you please also inform the proportion of targeted integration events over the total of transformant colonies? (the proportion of “nourseothricin-resistant and phleomycin-sensitive” to “total nourseothricin-resistant”; e.g. 6 colonies TI1–TI6 were obtained from how many total colonies selected for nourseothricin). This would help illustrate the extent to which counterselection is needed; and could help compare the transformation efficiency to other protocols in Bacidomicetes which report efficiency as targeted events over total transformant colonies (e.g. as table in Alberti et al 2020). Is the efficiency of targeted integration on total transformant colonies good enough for PCR screen of nourseothricin-resistant colonies alone? If the background of phleomycin-sensitive exists, is it due to random integration or single crossover recombination? If this information is known, adding it to the manuscript could help the community optimise the strategy in future works.

Minor comments

1. In fluorescence photography, please clarify the wavelength of the excitation light and the bandwidth of the filters used. Also, the methods describe that “The transformants and wild type H4–8” were assessed for fluorescent photography, however, negative controls are not present on the figure panel. Could they be added to contextualise background fluorescence? It would be good to also describe the method to spot the colonies for imaging.

2. Please clarify the genome accession ID for “scaffold 6: 2369122–2369196 “(E.g GeneBank ID; strain).

3. As testing targeted integration with CRISPR/Cas9 in WT background should be technically feasible (even if at low efficiency as in Vok et al 2019), the comparison in fluorescence could be performed within a single parental genetic background. This control could complement the current analysis which is only compares between strains with different parental genetic background (WT parent strain for random integration and ΔKU parent strain for targeted integration).

4. Plasmid construction method section: Is pPV040 a plasmid previously described in the literature? If so please cite the reference. Otherwise, could you please specify the origin of

the tubulin promoter? Is it from *S. commune*? Is dTomato amplified from any previously published construct (to link codon usage, etc)? It is noted that plasmid pPV033 ID#178359 is not yet on Addgene, is the code correct? it will effectively be a useful resource for the community to have on Addgene.

5. In “pPV048 was linearized with NdeI” could you please clarify if the restriction sites are at the end of the homology regions? The linearisation of plasmid template seems to be a change in the method to Vonk et al 2019, could you comment on this?

6. The sequence of the sgRNA spacer and design considerations are not currently present in the manuscript, could you please include this in the methods (coordinates or spacer sequence could be described besides the primers). Was off-target score accounted for in the design? Which program was used? In particular in the methods the expression “sgRNA was designed on the 75 bp” can be reworded for clarity (as the concept of 75 bp deletion is introduced later in the main text). Chromosomal coordinates for protospacer could be added otherwise.

7. Please add references in sentence “Previously, homologous recombination (which is required for targeted integration) was not efficient enough in *S. commune* to make this a feasible strategy.”

8. Are screening PCRs performed in isolated gDNA? Is this from primary colonies or after another round of selection? More description of methods can help.

Points not addressed in this review report: The *Schizophyllum commune* literature completeness in the introduction is not assessed due to reviewer’s lack of specific expertise on this species.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and does the work have academic merit?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbial synthetic biology and genetics. Expertise in developing CRISPR tools and systems for targeted gene integration and heterologous expression in filamentous fungi, which are relevant to this review report.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 14 March 2022

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Erika Kothe
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The article by Ohm *et al* shows a nice piece of work that will certainly accomplish easier knock-in with basidiomycetes, specifically of course the model Schizophyllum commune. It is nicely written and contains all the necessary information. There is, however, one shortcoming that should be addressed by the authors:

The authors nicely show that the span of expression is lower as compared to ectopic integration. That is to be expected. However, expression from the same site after targeted integration still varies among transformants between 70 and 110 %. This is better, but urgently needs an explanation. At least in discussion, this definitely needs to be addressed and potential explanations are required. If possible at all, the reason (e.g. chromatin modification in the integrated gene?) should be analyzed. As it stands now, the knock-in constructs still would need three to five transformants being investigated for a safe phenotypic characterization, and this is nowhere mentioned. For that matter, a second, rather not regulatory gene, should be knocked in as well to compare the level of expression.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, cell biology, molecular genetics, mycology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.