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Prostak *et al.*, *Access Microbiology* 2023;5:000566.v3
DOI 10.1099/acmi.0.000566.v3

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A guide to *Agrobacterium*-mediated transformation of the chytrid fungus *Spizellomyces punctatus*

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Abstract

Chytrid fungi play key ecological roles in aquatic ecosystems, and some species cause a devastating skin disease in frogs and salamanders. Additionally, chytrids occupy a unique phylogenetic position– sister to the well-studied Dikarya (the group including yeasts, sac fungi, and mushrooms) and related to animals– making chytrids useful for answering important evolutionary questions. Despite their importance, little is known about the basic cell biology of chytrids. A major barrier to understanding chytrid biology has been a lack of genetic tools with which to test molecular hypotheses. Medina and colleagues recently developed a protocol for *Agrobacterium*-mediated transformation of *Spizellomyces punctatus*. In this manuscript, we describe the general procedure including planning steps and expected results. We also provide in-depth, step-by-step protocols and video guides for performing the entirety of this transformation procedure on protocols.io (dx.doi.org/10.17504/protocols.io.x54v9d-d1pg3e/v1).

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary files. Supplementary materials include detailed steps for the entire protocol discussed in this manuscript. These steps can also be found on protocols.io (dx.doi.org/10.17504/protocols.io.x54v9dd1pg3e/v1).

INTRODUCTION

Chytrids are early-branching, largely aquatic fungi of great ecological importance [1]. While many chytrid species are free-living saprobes [2], others are deadly parasites of diatoms, algae, and some vertebrates [3]. One species, *Batrachochytrium dendrobatidis* (*Bd*), has garnered widespread attention as the 'frog-killing fungus', causing a deadly skin infection that is devastating frog populations around the world [4, 5]. Moreover, their phylogenetic position places them towards the base of the fungal lineage, having diverged before the diversification of the Dikarya (the group including yeasts, sac fungi, and mushrooms). This phylogenetic position, along with the retention of both fungal and ancestral traits like motile cilia (also called flagella) [6], make them especially useful for answering key evolutionary questions [1].

Despite their clear importance, little is known about the molecular and cell biology of chytrids. The major barrier for answering mechanistic questions in chytrids is a lack of genetic tools to use in the lineage. Recently, Medina and colleagues developed the first and only protocol to stably express transgenes in the chytrid species *Spizellomyces punctatus* (*S.p.*) using *Agrobacterium tumefaciens*-mediated transformation [7]. This technique opens the door to investigating chytrid pathogenesis and broadens researchers' abilities to explore the evolution of animals, fungi, and their unicellular relatives.

Chytrids have a biphasic life cycle, spending the first part of their life as a motile zoospore with a singular posterior flagellum and no cell wall. Motile zoospores eventually settle, retract their flagellum, build a cell wall, and grow to become sessile, reproductive sporangia. Sporangia undergo multiple rounds of mitosis without cytokinesis before dividing their cytoplasm to produce the next generation of mononuclear zoospores that are released back into the environment. *S.p.* undergoes a complete

Received 19 January 2023; Accepted 03 April 2023; Published 11 May 2023
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Keywords: Agrobacterium-mediated transformation; *Agrobacterium*; *Spizellomyces punctatus*; chytrid fungi; chytrids; transformation; genetic tools.
Abbreviations: A.t., agrobacterium tumefaciens; DS, dilute salts; h, hour(s); IM, induction media; LB, Luria-Bertani; OD, optical density; PCR, polymerase chain reaction; S.p., spizellomyces punctatus.
A supplementary file is available with the online version of this article.

life cycle from single zoospore to release of 20–50 zoospores in just 18–20 h when grown at 28°C on K1 media (w/v: 0.06% peptone; 0.04% yeast extract; 0.18% glucose; 1.5% agar). These simple growth conditions make *S.p*. an excellent organism for use in the laboratory.

S.p. transformation relies on *Agrobacterium tumefaciens (A.t*.), a plant pathogen that has been co-opted to genetically modify a wide variety of organisms. Infection by *A.t*. results in transfer of *A.t*. DNA into the plant genome that induces tumours and changes plant metabolism [8]. This organism and its infection strategy has been adapted for genetic manipulation of many plants and fungi [9, 10]. *S.p*. is currently the only chytrid that can be genetically modified by *Agrobacterium*-mediated transformation [7].

Here, we provide an overview of the protocol for *A.t*.-mediated transformation of *S.p*. A detailed collection of experiment steps, materials, timing, and precautions is available at protocols.io (dx.doi.org/10.17504/protocols.io.x54v9dd1pg3e/v1). A roughly 40 min video of the entire protocol is also associated with the entry on protocols.io to help demonstrate the entire process, focusing on the more intricate steps. We hope that this document and its accompanying materials will aid in the dissemination of knowledge at this pivotal point in the history of chytrid research and the broader field of evolutionary research.

ADVANCED PLANNING

Generally, *S.p*. transformation includes the following steps: 1) transforming *A.t*. with a plasmid of interest; 2) growing transformed *A.t*. to an OD660 of 0.6; 3) co-culturing *S.p*. zoospores and *A.t*. on a medium that induces the bacteria's virulence genes; 4) selecting for transformed *S.p*. using antifungal compounds; and 5) picking transformed colonies and culturing them.

This procedure involves many steps that span nearly a month and require significant advance planning. Fig. 1 outlines an efficient timeline for the entire procedure, including advance preparation of necessary materials. Of particular importance is allowing sufficient time for *A.t*. growth; *A.t*. lawns, colonies, or liquid cultures should not be used for any step of this procedure unless they have been growing at 28°C for at least 48 h. These conditions are based on our laboratory set up, and further optimization from the community is welcomed. The number of each type of plate required per plasmid to be transformed is outlined in Table 1.

Several growth times must be considered when planning steps before and on transformation day. Prior to transformation, *S.p*. cultures must be semi-synchronized and grown on antimicrobial-free media for at least two generations. To synchronize the population, *S.p*. should be subcultured roughly 36 and 18 h prior to your planned transformation time. On transformation day, *A.t*. needs to grow to an OD660 of 0.6, which in our experience takes about 4 h. The timing for growth to the proper OD should be empirically tested for each laboratory. Additionally, harvesting *S.p*. zoospores on transformation day takes about 1 h and should be coordinated so that *A.t*. and *S.p*. zoospores are ready around the same time.

After transformation day, you will need to return 12–24 h later to seal and invert the co-culture plates. It will take roughly 4 days to see any growth on these co-culture plates. Selecting for transformants takes another 4–6 days before the appearance of *S.p*. colonies on selection media. Once colonies appear, amplification of the colonies to grow enough cells for downstream applications can take up to another week. Overall, it will take about 4 weeks to go from wild-type *A.t*. to stably transformed *S.p*. cultures. In our experience, this method is successful over 80% of the time.

METHODS

The protocols described in this article are published in detail on protocols.io (dx.doi.org/10.17504/protocols.io.x54v9dd1pg3e/v1) and are included in the supplement here for printing (File. S1, available in the online version of this article). A roughly 40 min video detailing the entire *S.p*. transformation protocol–from electroporation of *A.t*. to selecting for and culturing *S.p*. transformants–can also be found on protocols.io.

Growing liquid cultures prior to transformation day

We prepared competent *Agrobacterium tumefaciens* EHA105 (*A.t*.; GoldBio #CC-225–5×50) cells at 4°C with cold reagents, as described in Weigel and Glazebrook [11]. Briefly, we grew *A.t*. at 28°C for at least 48 h before harvesting the cells and washing three times with water. We resuspended cells into sterile 10% glycerol and transformed them with a plasmid of interest using a 2 mm cuvette and a GenePulser exponential decay electroporator (BioRad, USA) with the following settings: 25 µF, 200 Ω, 2.5 kV. We have had success with plasmids derived from the plasmid pPZP201-BK [12]. Cells recovered in SOC medium by shaking at 225 r.p.m. for 4 h at 28°C. We then plated the cells onto LB plates with selection antibiotics (we typically use 50 mg l^{-1} kanamycin) and grew at 28°C for 4 days or until individual colonies appeared. We picked colonies into 5 ml LB broth each with selection antibiotics and grew the cultures overnight at 28°C and shaking at 225 r.p.m. We recommend freezing 0.5 ml of this culture in 25% glycerol at −80°C. In case the *Spizellomyces* transformation is unsuccessful, you can streak from this stock for colonies of the transformed *A.t*. The rest of the culture we used in co-culturing of *A.t*. and *S.p*., described later.

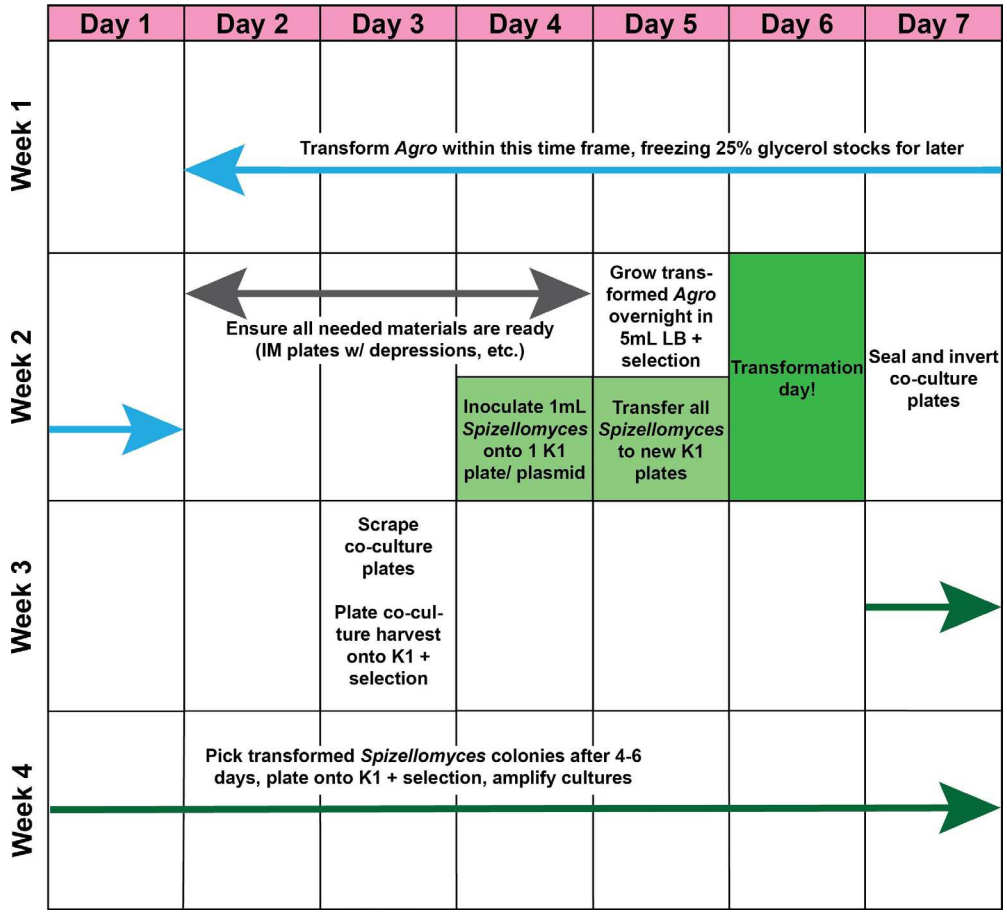

Fig. 1. Overview of *Agrobacterium*-mediated *Spizellomyces punctatus* transformation. A general timeline of the different steps involved in the transformation of *Spizellomyces punctatus* (*S.p.*) by *Agrobacterium tumefaciens (Agro, A.t.). A.t.* should generally be electroporated with the plasmid of interest at least 4 days prior to the planned transformation time to ensure that pickable colonies are obtained. All materials should be prepared 3–4 days prior to transformation day. *S.p.* should be subcultured onto antimicrobial-free K1 media 36 and 18 h prior to the intended transformation time. Following transformation day, seal and invert co-culture plates. About 4 days after transformation day, harvest the co-culture plates and select transformants by plating on selective K1 media. Cells will need to grow for 4–6 days after being plated onto selection media before colonies appear (if the transformation was successful). Overall, the process takes about 4 weeks from electroporating *A.t.* to having a stable culture of transformed *S.p.*

Table 1. The number and types of agar plates required per plasmid for *Agrobacterium*-mediated transformation of *Spizellomyces punctatus*

	no. of plates per plasmid				
Protocol Step	LB	LB+selection	K1	K1+selection	IM w/ depressions
A.t. Electroporation	1	1	–	–	–
Culturing S.p. 36 h before transformation	–	–	1	–	–
Culturing S.p. 18 h before transformation	–	–	1	–	–
Co-culturing A.t. and S.p.	–	–	–	–	1
Selecting for S.p.transformants	–	–	1	1	–
Picking colonies of transformants	–	–	–	one per four colonies	–
Total # of plates per plasmid	1	1	3	2	1

A.t., Agrobacterium tumefaciens; S.p., Spizellomyces punctatus.

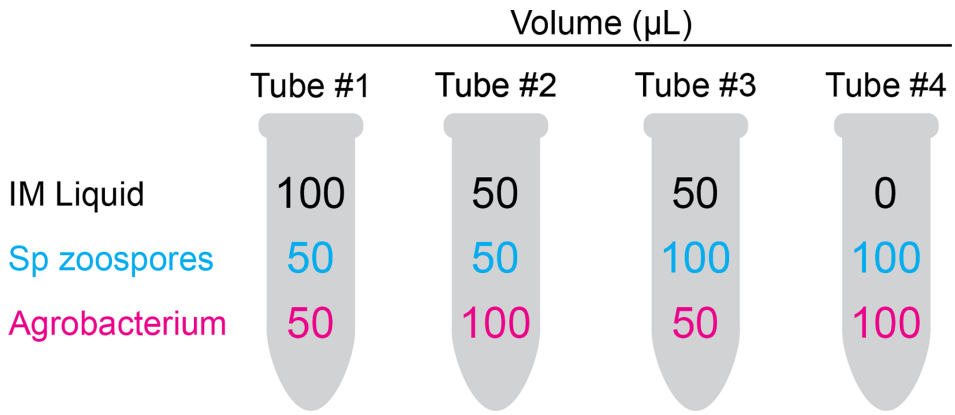

Fig. 2. Co-culture ratios of *Agrobacterium tumefaciens* and *Spizellomyces punctatus*. Using several ratios of *Agrobacterium tumefaciens (A.t.) to Spizellomyces punctatus (S.p.)* for co-culturing increases the chances of successful transformation. This is necessary due to the natural variation in zoospore release from day-to-day. Set up four 1.5 ml microcentrifuge tubes per plasmid with the indicated volumes of either IM liquid, *S.p.* zoospores (aim for between 1×10^6 and 1×10^7 cells ml^{-1}), and *A.t.* at an OD660 of 0.6. To prevent cross contamination of plasmids, fill the tubes in this order: IM then *S.p.* then *A.t.*

Culturing *Spizellomyces punctatus* prior to transformation day

Unless otherwise noted, we grew *Spizellomyces punctatus* Koch type isolate NG-3 Barr (*S.p.*; ATCC 48900) cultures at room temperature on K1 agar plates [0.06% bacto peptone (w/v; BD #211677), 0.04% yeast extract (w/v; Fisher #BP1422-2), 0.18% glucose (w/v; Sigma #G5767-5KG), 1.5% agar (w/v; Fisher #BP1423-500)]. About 36 and 18 h before the intended *S.p.* transformation time, we subcultured *S.p.* onto one fresh K1 plate per plasmid to be transformed.

Agrobacterium-mediated transformation of *Spizellomyces punctatus*

We diluted overnight *A.t.* liquid cultures to an OD660 of 0.15 in induction media [1 x minimal salts [13], 40 mM 2-(N-morpholino) ethanesulfonic acid (MES; Sigma #M2933-500G) pH 5.3, 10 mM glucose, 0.5% (v/v) glycerol (Fisher #G33-500), 200 µM acetosyringone (Sigma #D134406-5G)]. We then grew the cells to an OD600 of 0.6 by shaking at 225 r.p.m. for 4 h at 28 °C.

Meanwhile, we harvested *S.p.* zoospores by flooding culture plates with induction media (IM) or Dilute Salts (DS) solution [14] for 1 h before pooling all zoospores together. We then passed the suspension through a 40 µM mesh filter and then again through a sterile syringe filter with Whatman grade one filter paper. We aimed for a filtered zoospore concentration between 1×10^6 and 1×10^7 cells ml^{-1}, centrifuging the cells at 2000 rcf for 5 min and resuspending into IM if needed.

For each plasmid to be transformed, we mixed *A.t.* (at OD660 0.6) and *S.p.* zoospores (filtered) in four ratios in IM (Fig. 2), at a final volume of 200 µl. We then plated the total volume of each ratio onto one quadrant of an IM plate. To ensure a tight contact between the *A.t.* and *S.p.* cells, we made a roughly 1-inch diameter depression in each quadrant of an IM plate, created by gently pressing a warm, sterile, round-bottomed glass tube into the surface of the agar. We left the plates unsealed until the co-culture liquid dried (about 12–24 h) and then sealed and incubated them at room temperature for 4 days.

Selecting for *Spizellomyces punctatus* transformants

After the co-culture plates grew for 4 days, we rehydrated each quadrant with ~250 µl of DS. To harvest the cells, we scraped the surface of the agar with a sterile, single edged razor blade, washed the plate three times with 1 ml of DS, each time adding the liquid to the same 50 ml conical tube. We then brought the volume of the tube to 30 ml with DS. We mixed the cells by inverting the tube three times and vortexed it for 1–2 s to dislodge any *A.t.* still attached to *S.p.* We then pelleted the cells at 2000 rcf for 10 min and gently poured off the supernatant and resuspended the cells into 500 µl of DS. Then, we plated 200 µl of resuspended cells onto K1 plates with selection antimicrobials (300 mg l^{-1} hygromycin B to select for *S.p.* transformants, 50 mg l^{-1} carbenicillin and 50 mg l^{-1} tetracycline to prevent *A.t.* growth). Once the plates were dry, we incubated them in a humidity chamber at room temperature for 4 days until individual colonies appeared. The number of colonies varies; we typically obtain 5–100 *S.p.* colonies per transformation.

Picking colonies of transformed *Spizellomyces punctatus*

Once colonies of *S.p.* appeared on selection media, we picked and grew a few of them for downstream validation and analysis. We picked colonies by gently lifting them from the agar with a sterile 18G needle and resuspending each into 50 µl of DS. We gently broke up the colony by pipetting and then plated the colony suspension onto selective K1 media. To save time

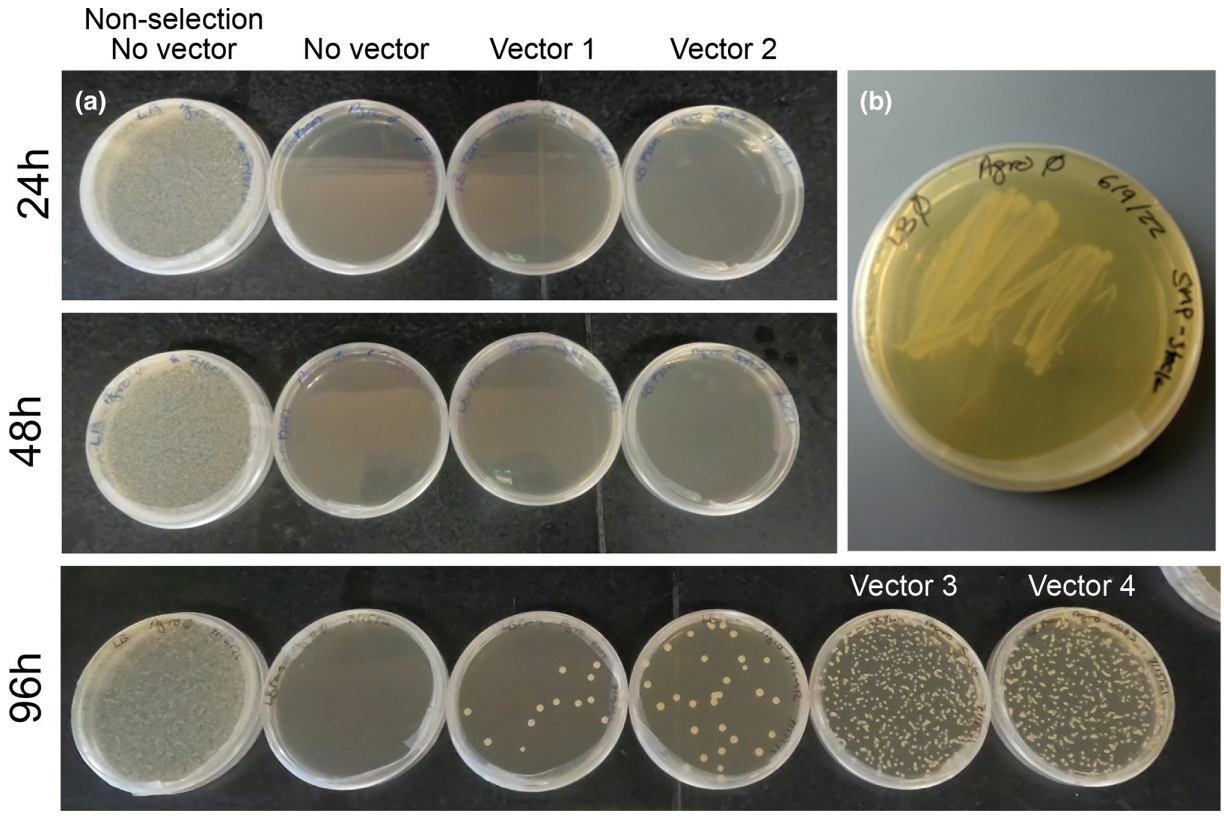

Fig. 3. Growth of *Agrobacterium tumefaciens* on LB media. (a) *Agrobacterium tumefaciens (A.t.)* was electroporated with no vector or vector 1, 2, 3, or 4. No vector controls were plated on non-selective and selective LB media. Cells electroporated with each vector were plated on selective LB media. Plates were incubated at 28°C and imaged at 24, 48, and 96 h post-plating to show the progression of colony growth. Different transformations yield varying colony morphologies. Visible growth does not typically occur until 72–96 h after plating. (b) Streak of wild-type *A.t.* on non-selective media. The overall appearance of growth on the plate is similar to that of streaks of transformed strains of *A.t.* on selective media.

and materials, we plated up to four colonies from the same plasmid transformation onto one quadrant each of a single plate. After 2–3 days we rehydrated each of these quadrants with 100 µl of DS and then transferred them to their own K1 plate. We continued subculturing until there was enough culture to freeze and also to continue with downstream procedures. The method used to confirm success of transformation depends on the downstream applications. We typically use PCR validation of the hygromycin resistance gene, but we also use western, northern, and Southern blotting and/or fluorescence microscopy if the gene of interest has a fluorescent tag [1].

EXPECTED RESULTS

Agrobacterium tumefaciens electroporation & growing liquid cultures prior to transformation day

Successful electroporation of *A.t.* is evidenced by the presence of colonies after 72–96 h on selective LB media (Fig. 3a). Colonies can vary in number and size, ranging from 0.1 to 1 mm. Make sure to plate a no vector electroporation sample on both selective and non-selective media controls. This step is to check if the cells survived the electroporation and to check for inherent selection resistance in the competent cells. When creating streaked plates from frozen stock of the desired *A.t.* strain, the plate should look similar to that in Fig. 3b. Liquid cultures of *A.t.* should be cloudy if growth was successful.

Culturing *Spizellomyces punctatus* prior to transformation day

Determining the health of the *S.p.* culture prior to transformation day is important. An unhealthy culture will not yield as many transformants. *S.p.* cultures are healthy if there are active, swimming zoospores and some free spaces between sporangia when viewed under a microscope.

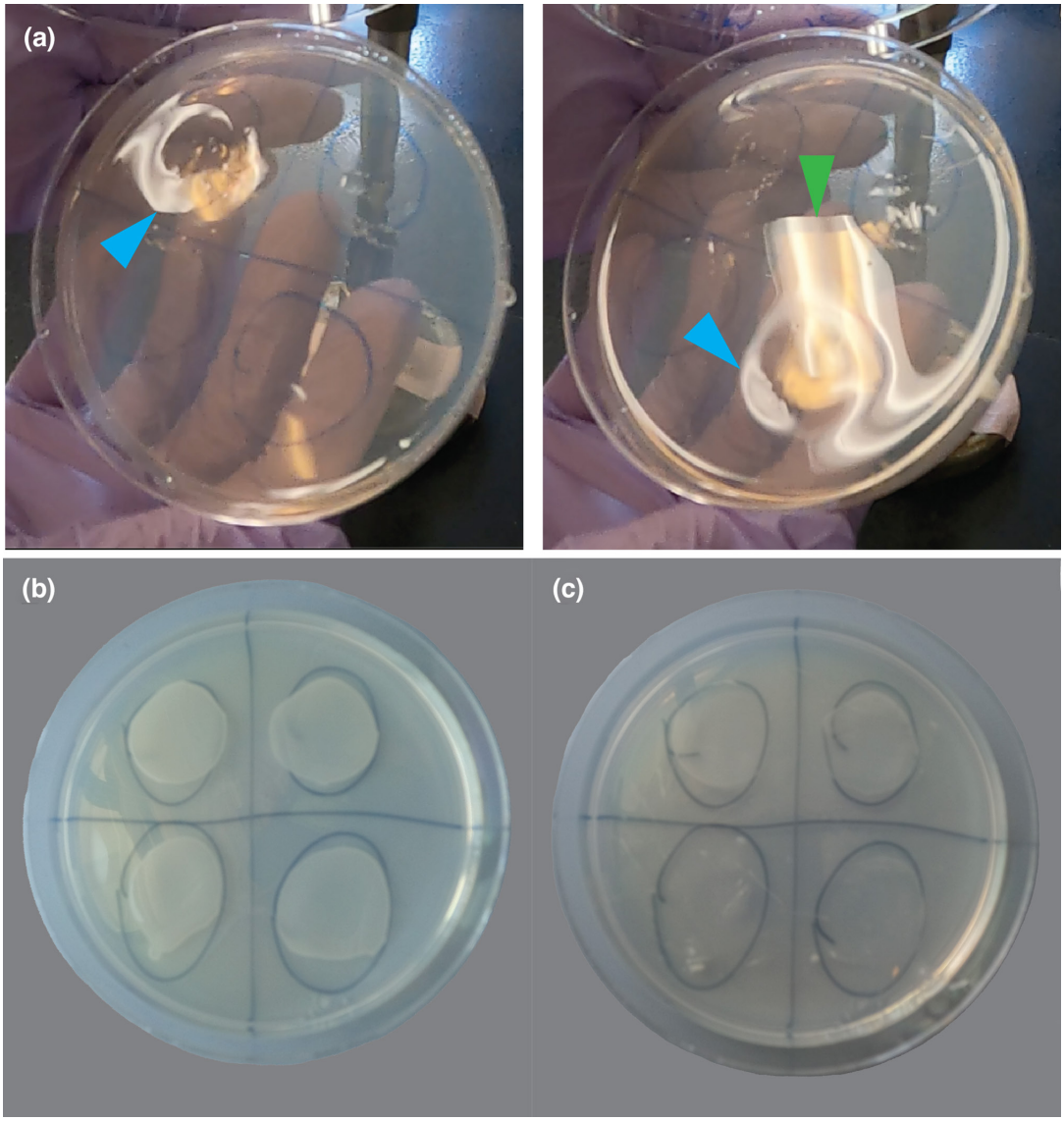

Fig. 4. Evaluation of depressions and co-culturing *Agrobacterium tumefaciens* and *Spizellomyces punctatus* on IM plates. (a) These ~1 inch in diameter depressions are made to ensure tight contact between *Agrobacterium tumefaciens* and *Spizellomyces punctatus* cells. As light hits depressed agar, it reflects around the curved perimeter (blue arrows), while light that hits non-depressed agar remains fairly linear (green arrow). This is a simple method to evaluate the size of the depressions created in the agar (see Methods and dx.doi.org/10.17504/protocols.io.x54v9dd1pg3e/v1). (b) Image of a co-culture IM plate after 4 days of incubation at room temperature. Growth appears as opaque areas on the agar. (c) Image of the same plate in (b) after harvesting cells by scraping with a sterile razor blade (see Methods and dx.doi.org/10.17504/protocols.io.x54v9dd1pg3e/v1). Nearly all of the opaque areas should be removed to increase chances of recovering transformed *Spizellomyces punctatus*.

Agrobacterium-mediated transformation of *Spizellomyces punctatus*

Creating depressions that can hold 200 µl of co-culture is important in increasing the chances of transformation success. Depressions in the fairly clear IM plates used for co-culturing *A.t.* and *S.p.* can be difficult to see. Holding the plate at an angle against the light allows for better detection of the depressions (Fig. 4a). Make sure to maintain sterility of the plate while doing this. The light will bend around the perimeter of the depression (Fig. 4a, blue arrows), but will stay nearly linear when hitting non-deformed agar (Fig. 4a, green arrows).

Once the depressions are the right size (about an inch in diameter and several millimetres deep), these IM plates will be used to co-culture *A.t.* and *S.p.* After 4 days at room temperature, the plates will be ready to harvest if opaque growth is present (Fig. 4b). After the harvesting process, nearly all of the growth should be removed from the plate (Fig. 4c).

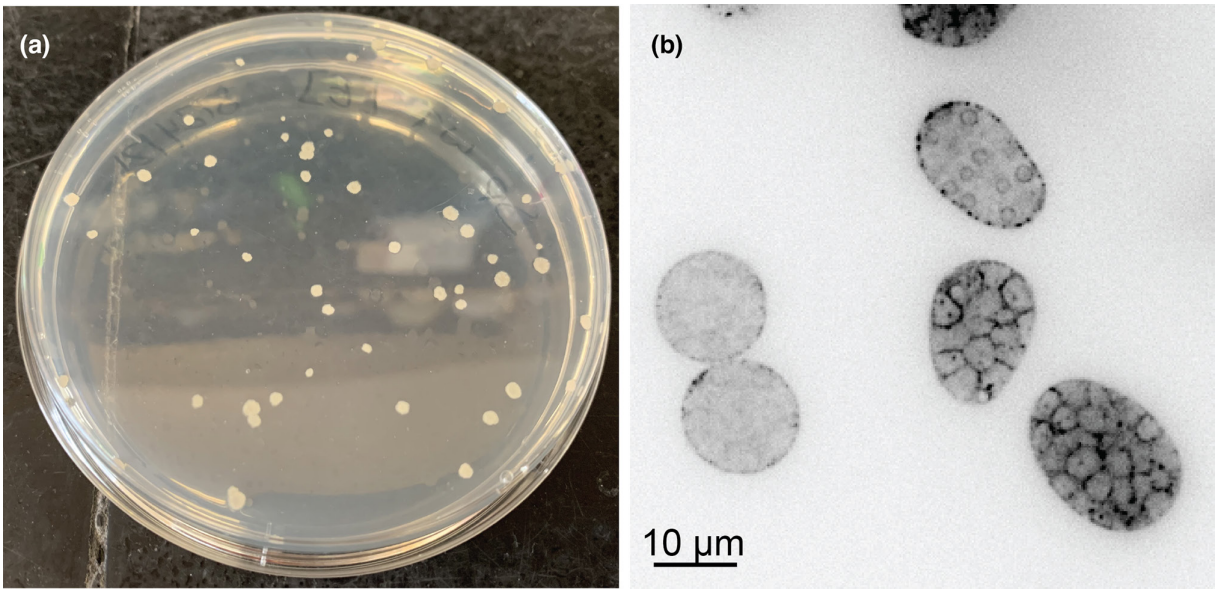

Fig. 5. Colonies of transformed *Spizellomyces punctatus* and a Lifeact-tdTomato strain. (a) Colonies of transformed *Spizellomyces punctatus* (*S.p.*), grown on selective K1 media for 4 days at 28°C. Colonies are rough, opaque and off-white. (b) Fluorescent images of a strain of *S.p.* stably expressing Lifeact-tdTomato that highlights polymerized actin (black).

Selecting for *Spizellomyces punctatus* transformants

After harvesting the co-culture, transformed *S.p.* can be selected for. If transformation was successful, colonies of transformants should appear on selective K1 media after about 4 days at 28°C (Fig. 5a). These colonies should be rough, opaque, and off-white, and can number from a few to hundreds.

Picking colonies of transformed *Spizellomyces punctatus*

Once a colony is picked and plated onto a new selective K1 plate, the resulting growth after about 4 days at 28°C should appear similar to the growth seen on the IM plates (Fig. 4b). When viewed under a microscope, zoospores should be swimming and there may be some larger-than-normal sporangia, this is a typical response to transformation for *S.p.* No bacteria should be present on the plates of isolated transformants.

Transformants should be verified through molecular analysis and microscopy. Here, we provide an example of *S.p.* transformed with LifeAct-tdTomato to confirm that the transformation was successful (Fig. 5b).

FUTURE DIRECTIONS

As the only genetically tractable chytrid species at the time of this publication, transformation of the free-living species *S.p.* is a vital part of the toolkit for studying chytrid cell biology, broadening the knowledge on an ecologically important group of fungi. The approach detailed here facilitates random chromosomal integration of a genetic cassette, which allows for overexpression of (fluorescent) fusion proteins and/or testing promoter activity. Random integration, however, does not lend itself easily to targeted gene disruption. For that approach, we are hopeful that the recent development of high-efficiency cargo delivery by electroporation may be used to develop targeted gene disruption [15].

Chytrid fungi are genetically diverse and include over a thousand species. Adapting transformation protocols to other chytrid lineages will enable new lines of research. For example, expanding the toolkit for genetic manipulation to the frog-killing chytrid *Batrachochytrium dendrobatids* and other parasites could reveal molecular mechanisms used by chytrids to parasitize their hosts. Moreover, we look forward to molecular genetic tools being developed for multiple chytrid lineages to facilitate testing hypotheses regarding the evolution of fungi, and answer other key questions about these fascinating and ecologically important organisms.

Funding information
This work was funded by the Gordon and Betty Moore Foundation (award #9337), the National Science Foundation (IOS-1827257), and a Pew Scholar award from the Pew Charitable Trusts to L.K.F.-L., who is a Canadian Institute for Advanced Research fellow in the Fungal Kingdom: Threats and Opportunities programme.

Acknowledgements
The authors would like to thank Dr Nicolas Buchler's lab for developing this transformational protocol and the team at Protocols.io for aiding in protocol formatting.

Author contributions
S.M.P. conceptualization, data curation, investigation, project administration, resources, validation, visualization, writing- original draft, writing- review and editing. E.M. methodology, validation, writing- review and editing. E.K. validation, writing- review and editing. L.K.F.-L. 7 Page 7 of 10 conceptualization, supervision, funding acquisition, project administration, writing- review and editing, nitpicking.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Medina EM, Buchler NE. Chytrid fungi. *Curr Biol* 2020;30:R516–R520.
2. Grossart H-P, Van den Wyngaert S, Kagami M, Wurzbacher C, Cunliffe M, *et al*. Fungi in aquatic ecosystems. *Nat Rev Microbiol* 2019;17:339–354.
3. Klawonn I, Van den Wyngaert S, Parada AE, Arandia-Gorostidi N, Whitehouse MJ, *et al*. Characterizing the "fungal shunt": parasitic fungi on diatoms affect carbon flow and bacterial communities in aquatic microbial food webs. *Proc Natl Acad Sci* 2021;118:e2102225118.
4. O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, *et al*. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* 2018;360:621–627.
5. Yong E. The Worst Disease Ever Recorded; 2019. https://www.theatlantic.com/science/archive/2019/03/bd-frogs-apocalypse-disease/585862/ [accessed 7 November 2022].
6. Venard CM, Vasudevan KK, Stearns T. Cilium axoneme internalization and degradation in chytrid fungi. *Cytoskeleton* 2020;77:365–378.
7. Medina EM, Robinson KA, Bellingham-Johnstun K, Ianiri G, Laplante C, *et al*. Genetic transformation of *Spizellomyces punctatus*, a resource for studying chytrid biology and evolutionary cell biology. *Elife* 2020;9:e52741.
8. Chilton MD, Drummond MH, Merio DJ, Sciaky D, Montoya AL, *et al*. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 1977;11:263–271.
9. Gelvin SB. Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol Mol Biol Rev* 2003;67:16–37, .
10. Michielse CB, Hooykaas PJJ, van den Hondel CAMJJ, Ram AFJ. Agrobacterium-mediated transformation as a tool for functional genomics in fungi. *Curr Genet* 2005;48:1–17.
11. Weigel D, Glazebrook J. Transformation of agrobacterium using electroporation. *CSH Protoc* 2006;2006:pdb.prot4665.
12. Hajdukiewicz P, Svab Z, Maliga P. The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant Mol Biol* 1994;25:989–994.
13. Hooykaas PJJ, Roobol C, Schilperoort RA. Regulation of the transfer of TI plasmids of *Agrobacterium tumefaciens*. *J Gen Microbiol* 1979;110:99–109.
14. Machlis L. Evidence for a cexual hormone in allomyces. *Physiol Plant* 1958;11:181–192.
15. Swafford AJM, Hussey SP, Fritz-Laylin LK. High-efficiency electroporation of chytrid fungi. *Sci Rep* 2020;10:15145.

Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000566.v2.1

Rubén de Dios; Brunel University London, Life Sciences, UNITED KINGDOM

Date report received: 03 April 2023
Recommendation: Accept

Comments: In this revised version of the manuscript "A guide to Agrobacterium-mediated transformation of the chytrid fungus Spizellomyces punctatus", the authors have addressed all the reviewers' comments and suggestions. Thus, this manuscript is now suitable for publication. I would like to thank the authors for their diligence in amending the manuscript. Congratulations!

Author response to reviewers to Version 1

We thank the editor and two reviewers for their efforts in reviewing our manuscript. We have addressed each of the comments in the revised manuscript as detailed below. Reviewer comments are in black and our responses in blue.

Reviewer 1:

This 'methods' paper presents a protocol for the transformation of the chytrid species Spizellomyces punctatus with T-DNA delivered from Agrobacterium tumefaciens. To date, S. punctatus is the only species in amongst a suite of early-diverging lineages of the fungi that can be stably transformed, a tool that can open up this and other species to direct testing of gene functions or other molecular biology discoveries.

1. Methodological rigour, reproducibility and availability of underlying data

The paper is structured as a conventional article that provides a brief overview of the methods, which include generating a suitable Agrobacterium strain, culturing bacteria and fungi, the transformation media and process, and then selection of transgenic S. punctatus lines. The previous studies have reported on the success of the transformation method (i.e. Medina et al. 2020). It would be interesting to know how often the authors have had success or failure using the method, and if it works on more than the single S. punctatus strain reported to date.

To date, we have been successful with this method on the strain of *Spizellomyces punctatus*that corresponds to the type isolate (i.e. Culture from the original material from original isolate from Koch 1957) used to describe the species and used to name the order Spizellomycetales (see. Barr, 1980 https://doi.org/10.1139/b80-276). We have tried other chytrids without success…at least not yet!

This protocol has been successfully reproduced in three different labs (Nicolas Buchler, NCSU; Lillian Fritz-Laylin, UMass Amherst; Tim Stearns, Stanford). Currently, we have more than a dozen different strains with a diversity of promoters, terminators and tags, including cytoskeletal components, centrosome components, myosin, calcium sensors, luciferase, halotag, SNAPtag and more. Cases in which we have been unsuccessful have usually been due to problems unrelated to the transformation protocol itself, but rather due to the specific DNA cassette being transformed, e.g. specific promoters.

2. Presentation of results

The steps for transformation are illustrated with four figures, and a fifth shows the outcome of transformation as colonies on a petri dish and a micrograph of a transformed line expressing a fluorescent protein. These will help readers trying the method for the first time.

We are glad the reviewer thinks this will be useful for readers.

3. How the style and organization of the paper communicates and represents key findings

One query on presentation is that in addition to the paper, there is also a 40 minute video that has been recorded and a more detailed set of methods that have been deposited to protocols.io. These are likely the primary resources investigators would first

use, rather than the paper, so it would be worth considering how best to integrate those resources into the manuscript, e.g. would this be through upload to the journal website or links off the paper?

We appreciate the suggestion on improving access to the resources associated with this manuscript. The DOI was previously included in the introduction, and now we have also added the DOI for the protocols.io entry into the abstract of the manuscript for easy reference.

4. Literature analysis or discussion

As a methods paper on the one chytrid that can be transformed, there is little need for extensive text. However, the Discussion section is brief. That paragraph can be summarized as suggesting the next steps are to use the method in S. punctatus and then develop the same transformation method in other chytrids. There is scope to consider the practicalities of the next steps, especially given it is now well over three years since the original transformation method was deposited in bioRxiv. As someone contemplating trying this method, a consideration would be why has it not been reported again since the first report?

We agree that the discussion section is more of a future directions section and have renamed it accordingly.

As to why there have been no follow up papers; we believe that this is primarily because the transformation protocol is based on Agrobacterium mediated transformation, which can be daunting to labs who do not have experience with this approach. This was the impetus for developing the video protocol. We hope that this will help the chytrid community adopt and adapt this protocol for their own uses.

Moreover, the transformation protocol was published right at the onset of the COVID-19 pandemic, a time in which it was difficult for laboratories to adopt whole new research trajectories. We trust that the patience of the reviewer will be rewarded with future publications.

Questions could be: Will ploidy or nucleus numbers of chytrid lineages or cell types, and the implications this would have on gene manipulation? That is, how easy would it be to generate lines homozygous for gene disruptions, or would something like RNAi have to be used to impair gene expression (and if so, do these species have the RNAi machinery)? Or is another alternative a gene editing approach (but that might then just need electroporation)? Will the same plasmids and selection media work for other species, or will they need changing, e.g. different promoters to drive the selectable marker? It seems that Swafford et al. 2020 Sci Rep might offer some answers.

We are delighted to hear that the reviewer is familiar with our electroporation paper! We have added the following to the discussion:

Line 224: "The approach detailed here facilitates random chromosomal integration of a genetic cassette, which allows for over-expression of (fluorescent) fusion proteins and/or testing promoter activity. Random integration, however, does not lend itself easily to targeted gene disruption. For that approach, we are hopeful that the recent development of high-efficiency delivery by electroporation may be used to develop targeted gene disruption (Swafford et al., 2020)"

5. Any other relevant comments

A few minor typographical or other points for consideration are as follows.

Lines 15 and 36: recommend editing the phrase 'yeasts, sac fungi, and their multicellular relatives' as this is an odd definition of the Dikarya.

We have reworded this to : "...sister to the well-studied Dikarya (the group including yeasts, sac fungi, and mushrooms) and related to animals…" and "...having diverged before the diversification of the Dikarya (the group including yeasts, sac fungi, and mushrooms)...."

Line 47: 'motile cilia' reflects my ignorance wherein I think of cilia on things like Paramecium and flagella on chytrids. Are these the same structures, and are there non-motile versions as well? Perhaps it would be good to expand the text here for those more familiar with the Dikarya.

Although flagella and cilia are the same structures, different research communities sometimes tend to predominately use one of these terms. We have indicated this in the text.

Line 137: add a space between 'acetyosringone (Sigma'.

We have fixed this typo.

Lines 156-157: 'conical'. Does this mean a conical flask or a 50 ml Falcon tube?

We have clarified this .

Throughout the text: check correct use of the micron symbol instead of 'u', e.g. on lines 154, 160, 168 and on figure 2 and 5B.

We have edited the text and figures

Line 207: delete 'of growth'.

We have fixed this typo.

Line 212: add italics to 'Sp'.

We have fixed this typo.

Line 264: missing species name.

We have fixed this typo.

Line 271: delete 'table of contents'.

We have fixed this typo.

Figure 1: spelling of 'selection'.

We have fixed this typo.

Line 358: spelling of 'co-culturing'.

We have fixed this typo.

Reviewer 2

The ms by Prostak et al. represents a detailed protocol and guide to Agrobacterium-mediated transformation of the chytrid fungus Spizellomyces punctatus as was published by the same group of authors before (in less practical detail) in Curr. Biol. (ref.1). This protocol will be helpful to scientists interested in applying this protocol, especially when not experienced in Agrobacterium-mediated transformation of other fungal species.

The results ms starts with a short general introduction to the field, followed by the step-by-step protocol. In the protocol I miss indications as to how many transformants are generally obtained, how presence of the T-DNA is assessed in the hygromycin resistant colonies obtained (by PCR?) and whether transformation is stable as indicated by T-DNA presence in the genome. In general, the presentation is clear.

We appreciate your points about quantity of transformants and transformant validation, and have added a few sentences about these points:

Line 164: "The number of colonies to expect varies, but typically 5- 100 *S.p.*colonies can be seen on a selection plate for one transformation."

Line 174: "The method used to confirm success of transformation depends on the downstream applications. We typically use PCR validation of the hygromycin resistance gene, but we also use western, northern, and Southern blotting and/or fluorescence microscopy if the gene of interest has a fluorescent tag [1]."

I have also the following detailed comments:

line 69: Although there are two papers indicating that sea urchin and human cells are transformed by Agro, these studies do not exclude that this occurred through uptake of DNA from lysed bacteria. Also they have never seen a follow-up and AMT is so far NOT used for animals. Therefore, in this ms it would be more in balance to refer to plants and fungi, for which AMT is in use worldwide.

An excellent point! We have reworded the sentence and removed the citations.

line 89: is growth at 28 C really necessary. In fact Agro grows even faster at 30 C, but transformation occurs preferably at lower temp (20-25 C).

The conditions described worked best for our incubator setup, timing, and conditions. We welcome further tests and optimization from the community and have added a sentence indicating this.

Line 89: "These conditions are based on our laboratory set up, and further optimization from the community is welcomed."

line 120: would more gentle shaking not be more appropriate immediately after electroporation?

In our experience the speed has not had a significant effect on the protocol.

line 136: 40M MES?

Great catch! Thank you. We have fixe this to the correct unit of mM

line 146: mixed instead of co-cultured. Co-cultivation occurs after this step.

We have fixed this typo.

lines 135-152 co-cultivation. Traditionally, co-cultivation of Agro and fungi is performed on membrane filters which are placed on the medium in the petri dish. Why did the authors chose to perform co-cultivation directly on the medium in a depression in the medium? This is much more laborious and maybe less efficient. Did they test filters and didn't the procedure work with filters?

Yes, we tested filters placed directly on the plate surfaces and also filters in which the mixture of sporangia and bacteria are concentrated into the filter through filtration to increase contact between cells (mounting the filter in a syringe holder and passing the mixture with a syringe). Contrary to hyphal fungi, the filter cannot be placed directly in selection plates afterwards if single colonies of transformants are to be retrieved. Chytrids do not form large hyphal colonies easily observed in the filter. This means sporangia still need to be retrieved from the filters. We found retrieving the sporangia from the filters to be cumbersome, hard to do reliably and reduced the yield of recovered sporangia and highly variable success in transformation (possibly by damage to the coenocytes; hyphal fungi are more robust and tolerant of hyphal rupture). In the end, depressions were simpler, faster, cheaper and more reliable in our hands. Nevertheless, we welcome further optimization from the community.

line 146/149 and throughout: here the abbreviation Agro is used instead of Agrobacterium in full. And also Sp instead of the full name of the fungus. This should be explained and be in accordance with journal style. I would prefer a similar abbreviation, if allowed. for example: A.t. for Agroabcterium tumefaciens and S.p. for the fungus.

We have changed all abbreviations to be consistent.

line 3 protocol4: DNA instead of DAN.

We have fixed this typo.

line 4 protocol4: acetosyringone is NOT a hormone, but a vir-inducer.

We have fixed this typo.

p11 line 10, 11: Carb and Tet abbreviations are not explained.

We have fixed this typo.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000566.v1.5

Rubén de Dios; Brunel University London, Life Sciences, UNITED KINGDOM

Date report received: 22 February 2023
Recommendation: Minor Amendment

Comments: In summary, the study presents a detailed description of method for Agrobacterium-mediated transformation of the chytrid fungus Spizellomyces punctatus. After the peer-review process, several changes have been proposed and suggested to improve the quality of the manuscript prior to publication. Please, consider all the reviewers' suggestions and comments thoroughly, especially those concerning indicating information about the transformation frequency, detection of transformant and stability of the modifications. Additionally, the reviewers have suggested some point to enrich the discussion section that may be of interest for prospective readers. Please provide a revised manuscript containing all changes and a point-by-point response to all the reviewers' comments within 1 month.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000566.v1.4

Anonymous.

Date report received: 17 February 2023
Recommendation: Minor Amendment

Comments: The ms by Prostak et al. represents a detailed protocol and guide to Agrobacterium-mediated transformation of the chytrid fungus Spizellomyces punctatus as was published by the same group of authors before (in less practical detail) in Curr. Biol. (ref.1). This protocol will be helpful to scientists interested in applying this protocol, especially when not experienced in Agrobacterium-mediated transformation of other fungal species. The results ms starts with a short general introduction to the field, followed by the step-by-step protocol. In the protocol I miss indications as to how many transformants are generally obtained, how presence of the T-DNA is assessed in the hygromycin resistant colonies obtained (by PCR?) and whether transformation is stable as indicated by T-DNA presence in the genome. In general, the presentation is clear. I have also the following detailed comments: line 69: Although there are two papers indicating that sea urchin and human cells are transformed by Agro, these studies do not exclude that this occurred through uptake of DNA from lysed bacteria. Also they have never seen a follow-up and AMT is sofar NOT used for animals. Therefore, in this ms it would be more in balance to refer to plants and fungi, for which AMT is in use worldwide. line 89: is growth at 28 C really necessary. In fact Agro grows even faster at 30 C, but transformation occurs preferably at lower temp (20-25 C). line 120: would more gentle shaking not be more appropriate immediately after electroporation? line 136: 40M MES? line 146: mixed instead of co-cultured. Co-cultivation occurs after this step lines 135-152 co-cultivation. Traditionally, co-cultivation of Agro and fungi is performed on membrane filters which are placed on the medium in the petri dish. Why did the authors chose to perform co-cultivation directly on the medium in a depresion in the medium? This is much more laborious and maybe less efficient. Did they test filters and didn't the procedure work with filters? line 146/149 and throughout: here the abbreviation Agro is used instead of Agrobacterium in full. And also Sp instead of the full name of the fungus. This should be explained and be in accordance with journal style. I would prefer a similar abbreviation, if allowed. for example: A.t. for Agroabcterium tumefaciens and S.p. for the fungus. line 3 protocol4: DNA instead of DAN line 4 protocol4: acetosyringone is NOT a hormone, but a vir-inducer p11 line 10, 11: Carb and Tet abbreviations are not explained

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000566.v1.3

Anonymous.

Date report received: 03 February 2023
Recommendation: Minor Amendment

Comments: This 'methods' paper presents a protocol for the transformation of the chytrid species Spizellomyces punctatus with T-DNA delivered from Agrobacterium tumefaciens. To date, S. punctatus is the only species in amongst a suite of early-diverging lineages of the fungi that can be stably transformed, a tool that can open up this and other species to direct testing of gene functions or other molecular biology discoveries. 1. Methodological rigour, reproducibility and availability of underlying data The

paper is structured as a conventional article that provides a brief overview of the methods, which include generating a suitable Agrobacterium strain, culturing bacteria and fungi, the transformation media and process, and then selection of transgenic S. punctatus lines. The previous studies have reported on the success of the transformation method (i.e. Medina et al. 2020). It would be interesting to know how often the authors have had success or failure using the method, and if it works on more than the single S. punctatus strain reported to date. 2. Presentation of results The steps for transformation are illustrated with four figures, and a fifth shows the outcome of transformation as colonies on a petri dish and a micrograph of a transformed line expressing a fluorescent protein. These will help readers trying the method for the first time. 3. How the style and organization of the paper communicates and represents key findings One query on presentation is that in addition to the paper, there is also a 40 minute video that has been recorded and a more detailed set of methods that have been deposited to protocols.io. These are likely the primary resources investigators would first use, rather than the paper, so it would be worth considering how best to integrate those resources into the manuscript, e.g. would this be through upload to the journal website or links off the paper? 4. Literature analysis or discussion As a methods paper on the one chytrid that can be transformed, there is little need for extensive text. However, the Discussion section is brief. That paragraph can be summarized as suggesting the next steps are to use the method in S. punctatus and then develop the same transformation method in other chytrids. There is scope to consider the practicalities of the next steps, especially given it is now well over three years since the original transformation method was deposited in bioRxiv. As someone contemplating trying this method, a consideration would be why has it not been reported again since the first report? Questions could be: Will ploidy or nucleus numbers of chytrid lineages or cell types, and the implications this would have on gene manipulation? That is, how easy would it be to generate lines homozygous for gene disruptions, or would something like RNAi have to be used to impair gene expression (and if so, do these species have the RNAi machinery)? Or is another alternative a gene editing approach (but that might then just need electroporation)? Will the same plasmids and selection media work for other species, or will they need changing, e.g. different promoters to drive the selectable marker? It seems that Swafford et al. 2020 Sci Rep might offer some answers. 5. Any other relevant comments A few minor typographical or other points for consideration are as follows. Lines 15 and 36: recommend editing the phrase 'yeasts, sac fungi, and their multicellular relatives' as this is an odd definition of the Dikarya. Line 47: 'motile cilia' reflects my ignorance wherein I think of cilia on things like Paramecium and flagella on chytrids. Are these the same structures, and are there non-motile versions as well? Perhaps it would be good to expand the text here for those more familiar with the Dikarya. Line 137: add a space between 'acetyosringone (Sigma'. Lines 156-157: 'conical'. Does this mean a conical flask or a 50 ml Falcon tube? Throughout the text: check correct use of the micron symbol instead of 'u', e.g. on lines 154, 160, 168 and on figure 2 and 5B. Line 207: delete 'of growth'. Line 212: add italics to 'Sp'. Line 264: missing species name. Line 271: delete 'table of contents'. Figure 1: spelling of 'selection'. Line 358: spelling of 'co-culturing'.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000566.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000566.v1.2