Pursuit of chlorovirus genetic transformation and CRISPR/Cas9-mediated gene editing

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Introduction

Research examining chloroviruses has provided many unexpected findings and concepts to the scientific community over the past 40 years [1]. However, despite these major achievements, no transformation system has been developed that allows the genetic modification of the large dsDNA viruses that infect certain unicellular *Chlorella*-like green microalgae. This transformation bottleneck creates a significant handicap in exploration of chlorovirus genomes that range from 290 to 370 kb and that encode many unidentified proteins. The ability to genetically engineer chloroviruses, in order to study and eventually manipulate their biochemical pathways, would greatly enhance the utility of microalgae-chlorovirus counterparts as scientifically and industrially important organisms. Considering that no reliable current reverse genetics system exists for either *Chlorella variabilis* NC64A (hereafter NC64A) or chloroviruses, we are equally limited in the capacity to either characterize gene function or exploit unique virus-encoded proteins. With the advent of CRISPR technology and the ongoing discovery of new giant viruses and their annotated genomes, we are armed with resources that have yet to be married.

A significant barrier to genetic transformation of chloroviruses is the inaccessibility of its host, NC64A, to DNA or protein uptake. Genetic engineering of microalgal strains is difficult due to the great diversity of species with a variety of cell sizes, cell wall structures and composition and, likely, unique responses to foreign DNA [2]. Like plant cells, *Chlorella* cells are surrounded by a rigid outer cell wall composed of polysaccharides with a variety of sugars as well as lesser amounts of protein and lipid that presumably makes them more difficult to transform [3]. DNA delivery can be challenging since DNA has to be transferred through the cell wall, plasma membrane and nuclear membrane. Moreover, the cells must be able to survive the chemical or

mechanical treatments involved. Therefore, individualized protocols are needed for specific strains and, thus, a broad range of genetic transformation methods must be designed and tested.

Because microalgal cells are not able to take up exogenous DNA by nature, several genetic techniques have been developed for this purpose. Among transformation methods for the delivery of exogenous DNA, the most common techniques are electroporation, ballistic systems, agitation with glass beads and *Agrobacterium tumefaciens*-mediated transformation [4-7]. Although, most of these techniques have been proven to work with great success in such model algal strains as *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Scenedesmus*, *Ankistrodesmus* and some *Chlorella* sp. [8, 9], there is a lack of efficient and stable transformation techniques that can be applied to a broader range of microalgae strains.

In earlier reports of successful genetic transformation of specific *Chlorella* species, various methods were developed including the use of glass beads [10], *Agrobacterium tumefaciens*-mediated transformation [11-13], PEG [14], protoplasting [15, 16], and electroporation [17-19]. Electroporation has become the favored tool for DNA delivery and genetic transformation of several microalgal species including *Chlamydomonas reinhardtii* [4], *Scenedesmus obliquus* [20], and *Nannochloropsis* sp. [21]. However the protocol optimization is often challenging, time-consuming and, most importantly, only proven in selected *Chlorella* sp. (*C. ellipsoidea*, *C. vulgaris, C. minutissima, C. zofingiensis* and *C. pyrenoidosa*) [22]. Microalgal species display a wide spectrum of resistance to transformation often based on differences in their ability to take up and incorporate exogenous molecules into their genomes. Moreover, cell viability can decrease rapidly when high voltages are needed and, likewise, increases in DNA fragment lengths can also affect transformation efficiencies [23].

Less traditional methods of microalga-transformation are being explored with some success. Karas *et al.* (2015) [24] and Diner *et al*. (2016) [25] showed that episomal plasmids containing a yeast-derived centromeric sequence CEN6-ARSH4-HIS3 can be transferred by conjugation from *E. coli* strains to the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*. Recently, Munoz *et al.* (2019) [26] also reported an efficient and stable transformation of the green microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans* by transferring exogenous DNA from *E. coli* via conjugation.

CRISPR/Cas systems have been widely used to manipulate the genomes of both freshwater and marine microalgae [27]. In particular, there are a number of reports in which Cas9/sgRNA-ribonucleoproteins-based approaches have been used for algal genome engineering. For example, the Cas9 protein and sgRNA are preassembled *in vitro*, and directly delivered to algal cells either via electroporation or by a biolistic method [28, 29]. In *C. reinhardtii*, Cas9/sgRNA RNP complexes were directly delivered to the cells by electroporation and created targeted mutations in multiple loci [28, 30]. RNP-based approaches have also been used to generate more robust strains of the industrial alga *Coccomyxa* as a biofuel cell factory [31].

In attempts to modify chlorovirus DNA, we tested previously described transformation protocols for other *Chlorella* sp. to deliver preassembled Cas9 protein/sgRNA RNPs inside NC64A cells prior to infection. As a gene to target for Cas9/gRNA RNP modification, we chose a virus-encoded putative glycosyltransferase gene because we believed we could develop a screening scheme to select cells bearing mutations in the glycosylation pattern of the virus and because, if our hypothesis for functional identity of the gene was correct, we wished to use such mutants to investigate the details of chlorovirus glycosylation. Specifically, we chose to target NC64A CA-4B virus-encoded gene, *034r*, which is a homolog of the prototype NC64A virus

PBCV-1-encoded gene *a064r*, that encodes a highly characterized glycosyltransferase with three domains involved in protein glycosylation: domain 1 has a *β*-L-rhamnosyltransferase activity, domain 2 has an α-L-rhamnosyltransferase activity, and domain 3 is a methyltransferase (MT) that decorates one position in the terminal α-L-rhamnose unit [32]. The mutant selection scheme was based on the observation that CA-4B mutants can be selected by rabbit polyclonal antiserum derived from serologically distinct PBCV-1 mutants that have a mutation in gene *a064r* [33], that in turn produces truncated surface glycans. This antibody-based selection scheme, therefore, permits discrimination between wildtype viruses with native glycans decorating the major capsid protein (MCP) and viruses carrying an *a064r* gene mutation (caused by Cas9/gRNA-directed gene editing) that produce a specific surface glycan variant.

The overall strategy to modify chlorovirus DNA involved testing a variety of transformation methods that could support the delivery of preassembled Cas9 protein-sgRNA RNP complexes to generate a targeted gene cleavage event in the CA-4B gene *034r*. Transformation methods that were investigated included protocols with cell wall-targeting enzymes, electroporation, silicon carbide (SiC) whiskers, and cell-penetrating peptides (CPPs). The RNA-directed selection of a specific 20–22 bp nucleotide sequence within the target gene *034r* by the Cas9/sgRNA complex allows the two nuclease domains of Cas9 to create a double stranded break (DSB) at a predetermined site within the gene of interest. Repair of the DSB by the error-prone nonhomologous end joining (NHEJ) DNA repair system can result in gene inactivation (i.e., gene knockout). In an even more powerful approach, replacement of the cleaved DNA segment with a closely related DNA fragment via homologous-directed recombination (HDR) can result in gene replacement (i.e., gene knockin) or nucleotide(s) substitution. Previously, it was demonstrated that single-stranded oligodeoxynucleotides (ssODNs) could provide ~100-fold lower levels of

nonhomologous integrations compared with double-stranded counterparts [34] while providing scarless genomic editing and reduced unwanted off-target cutting [35]. Thus, in an effort to increase efficiency and accuracy of targeted DNA editing and replacement in chloroviruses, we also attempted to co-deliver a DNA template in the form of ssODN (Fig 1) with Cas9/RNPs that targets a 20 bp sequence within the first ~40 nucleotides of the *034r* domain 1 coding region. Our guide RNA was designed so that successful incorporation of a ssODN-mediated HDR event would remove a native MscI restriction site in *034r* while simultaneously introducing at a separate site a specific premature stop codon – a double event highly unlikely to occur by spontaneous gene mutation.

Fig 1. sgRNA and ssODN designs for targeting CA-4B putative glycosyltransferase-gene *034r*.

Top: Cartoon rendering of CA-4B putative glycosyltransferase-gene *034r* composed of three domains. Middle: DNA sequence of the initial coding region of the *034r* gene with the target site for Cas9/gRNA binding, cleavage and editing highlighted in gray. Red TGG, PAM site; green triangles, Cas9 cleavage sites; underlined, MscI restriction site. Bottom: Design of ssODN to replace the dinucleotide, CT, with AA (green) in the target gene. The homology arms specific to the gene target are flanking the nucleotides designed to be changed (green font). Shaded red, newly created stop codon.

Methods

Alga growth conditions. NC64A cells were grown in Bold's basal medium (BBM) (3 mM $NaNO_3$, 170 μM $CaCl_2$ $2H_2O$, 304 μM $MgSO_4$ $7H_2O$, 431 μM K_2HPO_4, 1.3 mM KH_2PO_4, 428

μM NaCl, 12 μM Na_2EDTA, 2.2 μM $FeCl_3$ $6H_2O$, 1.2 μM $MnCl_2$ $4H_2O$, 220 nM $ZnSO_4$ $7H_2O$, 50 nM $CoCl_2$ $6H_2O$, 99 nM Na_2MoO_4 $2H_2O$, 6.4 μM $CuSO_4$ $5H_2O$, 184 μM H_3BO_3) modified by the addition of 0.5% sucrose and 0.1% peptone (MBBM) [36]. All experiments were performed with cells grown to early log phase (4 - 7 × 10^6 cells/mL). Cell cultures were shaken (200 rpm) at 26 °C under continuous light.

***In vitro* Cas9/gRNA-directed DNA cleavage assay.** Production of chloroviruses PBCV-1, CA-4B and DNA isolation were performed as described [37]. An 835 bp CA-4B DNA target for Cas9/gRNA cleavage was PCR-amplified and purified using 2% agarose gel electrophoresis (DNA sequences for primers used for DNA amplification are provided in the legend to Fig S1). Purified *Streptococcus pyogenes* strain Cas9 (SpyCas9) and *Lachnospiraceae* bacterium Cpf1 (LbCpf1) (200 nM) were independently preincubated with sgRNA (600 nM) in cleavage buffer [1× NEBuffer 3 (New England Biolabs, Ipswich, MA), 10 mM DTT, 10 mM $CaCl_2$] at 37 °C for 15 min. Target DNA (20 nM) was added to a final volume of 20 μL. Reactions were incubated at 37 °C for 1 h. DNA in cleavage reactions was purified by using a MinElute PCR Purification Kit (Qiagen), resolved by size on a 2% agarose gel and imaged on Gel Doc XR+ and ChemiDoc XRS+ systems (Biorad).

Fig S1. *In vitro* cleavage assay. The target *034r* locus from NC64A virus CA-4B was PCR-amplified and incubated with preassembled Cas9 and sgRNA RNP complexes *in vitro*. The complete *in vitro* cleavage of the target locus confirmed active RNP formation. PCR product (835 nucleotides) was amplified using primers upstream and downstream of domain 1. Arrowheads indicate cleaved products. L, molecular size ladder. Forward PCR primer DNA sequence:

GCGGTGTTCTCTAAATTACC. Reverse PCR primer DNA sequence: CCAGTTGCTACCATCTCC.

ssODN construct. We used tandem co-delivery of a DNA template in the form of ssODN with Cas9/sgRNA RNPs in attempts to achieve HDR. The ssODN was 80 nt long, designed with homology arms extending 40 nt upstream and downstream of the sgRNA target in CA-4B *034r*, respectively (Fig 1). The ssODN contained two critical elements that permitted selection when incorporated into a mutant virus: (1) a point mutation (T to A) that converts a native MscI restriction site TGGCCA to AAGGCC, and (2) a separate site nucleotide substitution (C to A) that introduces a premature stop codon (TAA). A successful HDR event would remove the MscI restriction site and allow initial verification of HDR-mediated insertion by simply treating PCR amplicons with MscI and showing loss of the restriction site near the site of Cas9/RNP-mediated DNA cleavage. Amplicons were subsequently sequenced to determine if Cas9/gRNA- and ssODN-directed nucleotide replacement had occurred. The simultaneous presence of a premature stop codon would eliminate translation of the glycosyltransferase enzyme and result in a shortened glycan at the MCP surface of newly formed viruses. Such mutants could be discriminated from wild-type viruses in the antibody-based selection scheme described below.

CA-4B mutant selection using mutant glycan-specific antibodies. NC64A cells were infected with CA-4B (MOI 5) and incubated for 12 h (detailed below). Site-directed mutant viruses were selected with anti-Rabbit IgG magnetic beads (RayBiotech) according to the manufacturer's protocol (Fig 2A). In brief, rabbit polyclonal antiserum prepared against wild-type PBCV-1 (which also binds CA-4B) and collected during previous antibody studies [33], was added to a virally-induced cell lysate (see details below) and allowed to bind wild-type virus for 1 h. Goat anti-Rabbit

IgG magnetic beads were incubated with the rabbit antibody solution for 30 min and then separated using magnets. The unbound viruses were collected and subsequently incubated for 1 h with rabbit polyclonal antiserum derived from serologically distinct PBCV-1 mutants that have a mutation in gene *a064r* (homologous to CA-4B gene *034r*) that produces a specific truncated surface glycan. Goat anti-Rabbit IgG magnetic beads were incubated again with the rabbit antibody solution for 30 min and then separated with magnets. After the unbound particulates were washed from the beads, the bound mutant antibodies were eluted from the beads using the elution buffer. The beads were then magnetically separated from the eluted solution. The eluted antibodies coupled to mutant viruses were removed manually and the viruses were then plaque assayed. Individual plaques were selected, and target DNA regions were PCR amplified with specific primers. The PCR products were size-verified using agarose gel electrophoresis, eluted from the gel and sequenced using the Sanger method (GENEWIZ).

Fig 2. Recovery of CA-4B site-directed mutants by RNP-targeting.

(A) Workflow of antibody selection for mutant chloroviruses. Following incubation of macerozyme-treated NC64A cells with Cas9/gRNA RNPs targeting the *034r* gene, cells were infected with CA-4A (MOI 5). Rabbit polyclonal antiserum (PBCV-1 antibody, green) was added to the viral-induced cell lysate to interact with wild-type viruses. Goat anti-Rabbit IgG magnetic beads were incubated with the rabbit antibody solution and then separated using magnets. The unbound viruses were collected and subsequently incubated with antibody derived from serologically distinct PBCV-1 mutants (antigenic mutant antibody, red) that have shortened surface glycans. Magnetic beads were incubated again with the antibody solution and then separated using magnets. Bound mutant viruses were eluted from the beads and plaque assayed.

(B) Following antibody selection for mutant CA-4B viruses, 13 plaques were recovered. Domain 1 of *034r* from each virus isolate was sequenced. (C) Two indels (red) in *034r* from two CA-4B variants (1 and 2) were detected and sequence verified for site-directed mutagenesis by preassembled Cas9 protein-sgRNA RNPs in the PBCV-1 *a064r* homologous gene. PAM site, gray. Green triangle, Cas9/gRNA cleavage site. WT, wildtype sequence.

Cell wall-degrading enzymes. Cellulase, chitinase, chitosanase (25.9 U/mL), drieselase, β-glucosidase, β-glucuronidase (140 U/mL), hyaluronidase, laminarinase, lysozyme, lyticase, macerozyme, pectinase (3,000 U/mL), pectolyase, sulphatase (3.37 mg/mL), and trypsin were purchased from Sigma Aldrich (St. Louis, MO). Zymolyase (10 mg/mL) was purchased from ZymoResearch (Irvine, CA). Macerozyme was purchased from RPI corp (Mt Prospect, IL). Stock concentrations of enzymes were 20 mg/mL unless otherwise noted.

Assay of enzyme inhibited cell growth. To assay growth inhibition due to enzymatic activity, 200 µL of algal cells normalized to an OD_{750} of 1.0 was mixed with 4 mL of media containing 7.5 g/L agar, which was at 42 °C and poured on a petri plate containing 15 g/L agar. Once hardened, 10 µL of enzyme stock was then spotted on the top agar and plates were incubated in the light at 26 °C for 5 days. As a negative control for enzymatic activity, enzymes were heat denatured at 100 °C for 10 min and spotted onto plates.

NC64A transformation following enzymatic digestion of cell walls. For cell wall digestion, one mL of cell culture of NC64A (5×10^6 cells) was centrifuged at 8,000 rpm for 5 min and the pellet was resuspended in the same volume of MBBM in the presence of cell wall-degrading enzymes (Table 1) or MBBM only. The culture was incubated for 24 h at 25 °C in continuous light. NC64A cells were centrifuged and resuspended in a solution (0.8 M NaCl and 0.05 M $CaCl_2$). Prior to

NC64A cells being tested for their ability to be transformed with Cas9/sgRNA RNPs, all treated cells were previously infected with virus and analyzed by plaque assay to confirm treated cells retained their permissive qualities required for virus attachment and infection. To generate target-specific site-directed mutants using RNP complexes in NC64A, enzyme-treated and nontreated cells were transformed with either Cas9 or Cpf1 (200 μg) premixed with *034r*-targeting sgRNA (140 μg). Cas9 and Cpf1 proteins in storage buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerol) were mixed independently with sgRNA dissolved in nuclease-free water and incubated for 10 min at room temperature. The mixture of cells and Cas9 (or Cpf1)/sgRNA RNPs was incubated for 15 min at room temperature. Cas9(Cpf1)/sgRNA-transformed, enzyme-treated NC64A cells were then infected with CA-4B and incubated overnight. Virus mutants were selected using the same antibody selection assay previously described. DNA from virus plaques were analyzed by PCR and sequenced to detect indel mutations caused by NHEJ. In experiments testing for ssODN-mediated HDR, *034r* gene PCR amplicons were first tested for sensitivity to cleavage by MscI prior to DNA sequencing.

Table 1. Growth inhibition of *Chlorella variabilis* NC64A by a variety of enzymes.

Enzyme	NC64A
Cellulase	–
Chitinase	+ + +
Chitosanase	+
Driselase	–
β-Glucosidase	–
β-Glucuronidase	–
Hyaluronidase	–
Laminarinase	+ + +
Lysozyme	+ + +
Lyticase	+ + +
Macerozyme	+ + +
Pectinase	+ + +
Pectolyase	–
Sulphatase	+
Trypsin	–
Zymolyase	–

–, no growth inhibition; +++, complete growth inhibition

Flow cytometry. Cell permeability assays were performed on the BD FACSAria cell sorter using ATTO™ 550 labeled Alt-R™ Cas9 tracrRNA from IDT (Coralville, IA). The 67mer Alt-R™ CRISPR-Cas9 tracrRNA has a ATTO™ 550 fluorescent dye attached to the 5' end. The fluorescent dye allows for an optical analysis of transfected cells and cell sorting by FACS. During data acquisition, algal cells were positively defined by their chlorophyll autofluorescence. A minimum chlorophyll autofluorescence was set to eliminate potential false positives from bacteria and debris present in the culture. One μL of ATTO™ 550-labeled tracrRNA (200 uM) was added, incubated for 2 min, and loaded on the BD FACSAria where 20,000 cells were imaged. Samples were excited using a 488 nm laser and 660–740 nm (chlorophyll) and 480–560 nm (ATTO) emission data as well as bright field image data were collected. Populations were gated for in-focus cells and analyzed for permeability to labeled tracrRNA.

NC64A transformation using SiC whiskers. For each transformation, NC64A cells were concentrated and resuspended in 800 µL fresh MBBM (5×10^7 cells) in a 1.5 mL microfuge tube and mixed with 50 mg sterile SiC whiskers and preassembled Cas9/sgRNA RNP (armed with the protospacer sequence targeting domain 1 of CA-4B *034r*) in the presence and absence of the ssODN shown in Fig 1. Samples were agitated by a vortex mixer at top speed for 2 min, stopping briefly every 10 s. Immediately after vortexing, samples were diluted with 200 µL PEG (20% PEG 8000) reaching a final volume of 1 mL, and infected with CA-4B virus as described above. Following overnight infection, virus mutants were selected using the same antibody selection assay previously described. DNA from virus plaques were analyzed by PCR and sequenced to detect indel mutations caused by NHEJ, while *034r* gene PCR amplicons were also incubated with MscI to screen for ssODN-mediated HDR.

NC64A transformation using electroporation. Purified Cas9 (100 µg) was preincubated at a 1:3 molar ratio with the *034r*-targeting sgRNA at 37 °C for 15 min to form RNP complexes. For transfection, 250 µL cell culture (2.5×10^8 cells/mL) supplemented with sucrose (40 mM) were mixed with preincubated RNPs, and 150 nM ssODNs. Cells were electroporated in 2-mm cuvettes (600 V, 50 µF, 200 Ω) by using Gene Pulser Xcell (Biorad). Immediately after electroporation, 800 µL of MBBM with 40 mM sucrose was added to the sample and the cells were infected with CA-4B (MOI 5). Following overnight infection, virus mutants were selected using the same antibody selection assay previously described. Virus plaques were analyzed by PCR and sequenced to detect indel mutations by NHEJ, while *034r* gene PCR amplicons were also incubated with MscI to screen for ssODN-mediated HDR.

Cell-penetrating peptides. Peptides were provided by Dr. Heriberto Cerutti (UNL) and Keiji Numata (RIKEN): $(BP100)_2K_8$ (KKLFKKILKYLKKLFKKILKYLKKKKKKKK, theoretical

pI/Mw: 10.75/3851.13 Da) and BP100$(KH)_9$ (KKLFKKILKYLKHKHKHKHKHKHKHKH, theoretical pI/Mw: 10.81/ 3809.71 Da) [38]. Peptide/RNP complexes were prepared by adding different amounts of each peptide to the Cas9/sgRNA RNP mixture at various ratios (0.1, 0.5, 1, 2, 5, 10, and 20). The solution was thoroughly mixed by repeated pipetting and allowed to stabilize for 30 min at 25 °C.

NC64A transformation using CPPs. Referring to a previous study [30], 10 µg of Cas9 and 12 µg of sgRNA were incubated at room temperature for 15 min. A prepared cell sample of 100 µl at 3×10^8 cells/mL was added to the incubated RNP, ssODNs, and gently mixed. Tested independently, $(BP100)_2K_8$ or BP100$(KH)_9$ [38] was added to the sample and mixed immediately. In a series of experiments, we observed that there was no significant difference between the two types of treatment with CPPs: (1) pre-incubation of RNP and CPP for the formation of complex, and (2) CPP and RNP added separately to the cells. After incubation of the cells mixed with the RNP and CPP for 30 min at 25 °C, trypsin was added, and the mixture was incubated for 15 min at 37 °C. The sample was washed by MBBM media and transferred to 10 mL of MBBM media and incubated for 16 h under dim light without shaking as a "recovery" step. Following incubation, cells were infected with CA-4B (MOI 5) and incubated overnight (24 h). Following overnight infection, virus mutants were selected using the same antibody selection assay previously described. DNA from virus plaques were analyzed by PCR and sequenced to detect indel mutations caused by NHEJ, while *034r* gene PCR amplicons were also incubated with MscI to screen for ssODN-mediated HDR.

***Agrobacterium* strain and vectors.** The binary vector pCAMBIA1303 containing a gfp:gusA fusion reporter, gene a*064r* from a PBCV-1 antigenic mutant referred to as EPA-1, and a selectable marker for hygromycin B resistance driven by the CaMV 35S promoter were used for

transformation. The binary vector was mobilized into *A. tumefaciens* strain LBA4404 by using the Biorad Gene Pulser Xcell electroporator (Hercules, CA) according to manufacturer's protocol. Transformed cells were aliquoted and maintained at -80°C in 25% (v/v) glycerol.

Antibiotic sensitivity test. The sensitivity of *A. tumefaciens* towards the antibiotic cefotaxime was tested by inoculating 200 µL of *Agrobacterium* culture (OD_{600} = 1.0) in 5 mL LB broth supplemented with varying concentrations of cefotaxime (0, 50, 100, 150, 200, 300, 400 and 500 mg/L) and the growth of *Agrobacterium* in each concentration was measured spectrophotometrically at OD_{600} after 2 days. The effect of the antibiotic cefotaxime on the viability of NC64A was accessed by plating a serially-diluted microalgae culture on solid MBBM supplemented with different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500 mg/L). The agar plates were incubated in the dark for 2 days at 25 °C before exposure to light and the number of surviving colonies from the dilution that produced less than 100 colonies was counted in duplicates after 2 weeks. To determine the minimum inhibitory concentration of hygromycin B, 1 x 10^6 NC64A cells were plated on solid MBBM medium supplemented with 500 mg/L cefotaxime and varying concentrations of hygromycin B (6, 8, 10, 12, 14, 16, 18, 20, 23 and 26 mg/L). Each treatment was tested in triplicate. The agar plates were incubated for 2 days in the dark at 25 °C before exposure to light and the number of surviving colonies was accessed after 20 days.

NC64A transformation using *Agrobacterium*. A general transformation procedure for NC64A was established based on work done by Kumar *et al.* (2004) [5] on transformation of *C. reinhardtii* with some adjustment as described here. Single colonies of *Agrobacterium* initiated from a frozen stock were used to inoculate 10 mL of LB supplemented with 5 mM glucose, 100 mg/L streptomycin and 50 mg/L kanamycin and grown overnight in a rotary shaker at 27 °C with shaking

at 200 rpm in the dark. Five mL of this overnight culture was used to inoculate 50 mL of the same medium and it was grown in the dark at 27 °C with shaking at 200 rpm until OD_{600} = 0.8–1.2. The bacterial culture was harvested by centrifugation and washed once with induction medium (MBBM plus 150 μM acetosyringone, pH 5.6) and diluted to a final density of OD_{600} = 0.5. Prior to co-cultivation, a total of 5 x 10^6 NC64A cells from a log-phase culture (OD_{600} = 0.5–1.0) were pre-cultured for 5 days on MBBM agarose plates at 25 °C and harvested with induction medium on the day of co-cultivation. The algal cell pellet was mixed with 200 μL of the bacterial suspension and plated on induction medium solidified with 1.2% (w/v) bacto-agar. Co-cultivation was performed for 3 days at 25 °C in the dark. Following co-cultivation, cells were harvested with MBBM supplemented with 500 mg/L cefotaxime in a total volume of 7 mL and incubated in the dark at 25 °C for 2 days to eliminate *Agrobacterium*. Visualization of *gfp* expression was performed using a confocal fluorescent microscope (Nikon A1R-Ti2 confocal system, UNL Microscopy Core facility). Subsequently, the remaining cells were plated on selective media containing 20 mg/L hygromycin B and 500 mg/L cefotaxime and incubated at 25 °C in the dark for 2 days before exposure to light. Resistant colonies were propagated on non-selective media and utilized for PCR analysis. Assays for detection of contaminating *Agrobacterium* were performed by growing cells on LB agar plates for at least 7 days at 25 °C in the dark.

Results

Chitinase, laminarinase, lysozyme, lyticase, macerozyme, and pectinase enzymes inhibit growth of NC64A. Inhibition of cell growth by an active preparation of cell wall-degrading enzyme suggests the enzyme is either degrading the cell wall during its construction or that the enzyme interferes with precursor generation prior to precursor assembly into the cell wall. From a

variety of enzymes tested (Table 1), chitinase, laminarinase, lysozyme, lyticase, macerozyme, and pectinase had the greatest negative effect on NC64A growth as judged by the size of the zone of inhibition around the spotted area. By comparison, chitosanase and sulphatase caused minor growth inhibition. Heat denatured enzymes did not impact growth in the assay, indicating the growth inhibition was due to the enzymatic activity alone and not from other potentially toxic components in the enzyme preparations.

Preassembled RNPs with fluorescently labeled tracrRNA enters enzyme-treated NC64A cells. Fluorescently labeled Alt-R™ Cas9 tracrRNA was used to evaluate cell permeability and visualized using flow cytometer (BD FACSAria). The resulting data were analyzed by setting a minimum ATTO™ 550 fluorescent intensity threshold, such that the majority of untreated cells (Fig 3A) had fluorescence intensity lower than the threshold. Cells with a fluorescent intensity above this threshold were considered permeable to the tracrRNA dye as indicated by cells pretreated with macerozyme (Fig 3B), or vortexed with SiC whiskers or electroporated prior to fluorescence-activated cell sorting (FACS) (Fig 3C and 3D, respectively). Cells contained 5.6%, 12.4%, and 18.6% of total sample florescence when treated with either macerozyme, vortexed with SiC whiskers, or electroporated, respectively. In contrast, cells not treated with enzymes contained less than 0.1% of total sample fluorescence.

Fig 3. FACS dot plot analysis of fluorescently labeled NC64A cells.

NC64A cells were visualized by intracellular incorporation of fluorescently labeled tracrRNA (ATTO™ 550), (A) without enzyme treatment, or prior to FACS were (B) macerozyme digested, (C) vortexed with SiC whiskers, or (D) electroporated. Cells were illuminated with a white LED for bright field and a 488 nm laser for fluorescence. Images were collected in the bright field and

480–560 nm emission (ATTO™ 550 fluorescence) channels. Cell populations with a high fluorescent intensity were gated for in-focus cells and analyzed for permeability.

Design, production, and testing of a sgRNA targeting a specific site in the CA-4B virus *034r* gene. A sgRNA was designed to cleave the CA-4B virus in the first domain of the glycosyltransferase gene *034r* at 27 nucleotides into the coding region (Fig 1). To ensure this molecule was active and accurate in cleaving target DNA when combined *in vitro* with *Streptococcus pyogenes* strain Cas9 (SpyCas9), the appropriate sgRNA fragment (denoted in Fig 1) was synthesized and tested for its ability to cut a PCR-amplified 835 bp fragment of the CA-4B virus first domain. The data of Fig S1 confirmed the ability of the *in vitro*-assembled Cas9/sgRNA RNP complex to accurately cleave the CA-4B viral DNA fragment into the expected 600 bp and 235 bp fragments.

Antibody-based selection and recovery of Cas9/gRNA RNP-induced chlorovirus mutants from macerozyme-treated NC64A cells. We have shown previously that CA-4B is recognized by antibodies to PBCV-1 and by antibodies to certain PBCV-1 antigenic variants (mutants) making such antibodies useful to isolate wild-type and mutant versions of CA-4B virus [39]. In addition, studies involving spontaneous mutant viruses have demonstrated previously that disruption of PBCV-1 *a064r* leads to viruses with an altered glycan attached to the MCP and that these mutants are antigenically different from wild type PBCV-1 [40]. In additional studies [33, 41], polyclonal antisera were prepared against these spontaneous mutants. This allowed us to screen for viruses containing a disrupted *034r* gene using antibody that specifically recognized viruses with a gene-inactivating mutation in the homologous *a064r* gene.

Macerozyme-treated NC64A cells were mixed with Cas9/sgRNA RNP complexes targeting *034r* and subsequently infected with CA-4B overnight. Following incubation of viruses recovered from ruptured cells with the serologically distinct antigenic antibody, the putative mutant precipitate was eluted, and plaque assayed (Fig 2A). The presence of mutations in the resulting plaques were subsequently verified by DNA sequencing of domain 1 from *034r* using appropriate PCR primers. Two independent plaques showed separate modifications adjacent to the Cas9/sgRNA cleavage site (a nucleotide substitution in DNA from one plaque and a nucleotide substitution in DNA from the other – Fig 2C). This result is fully consistent with that expected from site-directed mutagenesis targeted in the PBCV-1 *a064r* homologous gene, CA-4B *034r,* by our specifically designed Cas9/sgRNA RNP complex (Fig 1 and Fig 2). These observations suggest successful delivery into macerozyme-treated NC64A cells of preassembled Cas9/sgRNA RNPs that resulted in a frameshift mutation in the CA-4B-encoded gene *034r* of one viral progeny and a phenylalanine to leucine codon change in a separate viral progeny. Many attempts to obtain additional examples of gene editing with this particular Cas9/sgRNA RNP failed.

No evidence for HDR events using ssODNs. All attempts at producing nucleotide sequence replacement by homologous DNA recombination (HDR) using ssODNs to simultaneously create a premature stop codon and remove the native MscI restriction site in the *034r* gene were also unsuccessful. Amplicons of *034r* DNA fragments from recovered CA-4B viral plaques after antibody selection were incubated with MscI. Virus DNA was digested into two DNA fragments that were consistent in size with a MscI-digested amplicon thus suggesting an intact MscI site in each of the samples tested (restriction digestions not shown). The conservation of the restriction site provides no evidence for ssODN-mediated HDR using the present methods.

Antibiotic sensitivity and resistance of *Agrobacterium* and NC64A cultures. Prior to testing if NC64A cells could be genetically transformed using *A. tumefaciens*, we determined the levels of hygromycin needed to kill nontransformed NC64A cells and the levels of cefotaxime needed to rid cultures of *A. tumefaciens* following co-incubation of the algal and bacterial cells. Growth of *A. tumefaciens* was suppressed at a cefotaxime concentration as low as 50 mg/L (Fig 4A) whereas the growth of NC64A was found to be uninhibited in cefotaxime-supplemented media up to 1000 mg/L (Fig 4B). Thus, 500 mg/L of cefotaxime was selected for all experiments to ensure thorough elimination of *Agrobacterium* post-transformation. The lowest concentration of hygromycin B which completely inhibited the growth of NC64A was 20 mg/L (Fig 4C), and this concentration was used for subsequent selection of transformants. To verify successful electroporation of the pCAMBIA1303 binary vector carrying a kanamycin resistance gene (see below) into the *A. tumefaciens* strain to be used for transforming NC64A cells, colony PCR was performed. The data in Fig 5 verified that successful plasmid mobilization into *A. tumefaciens* cells had occurred.

Fig 4. The effects of antibiotics on *Agrobacterium* and NC64A.

(A) The effect of cefotaxime on *Agrobacterium* viability. The effect of (B) cefotaxime and (C) hygromycin on NC64A viability.

PCR-confirmed *Agrobacterium*-mediated transformation of NC64A cells. To determine if *A. tumefaciens* could be used to transfer T-DNA containing foreign genes into the genome of NC64A cells, we mixed the algal cells with *A. tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1304 containing the gfp:gusA fusion reporter, a hygromycin phosphotransferase (*hpt*) selectable marker driven by the CaMV35S promoter, and the mutant a*064r glycosyltransferase*

gene from a PBCV-1 antigenic mutant referred to as EPA-1 (EPA1-*a064r*). The mutant gene contains a missense mutation (C→T) at nucleotide position 236 resulting in an amino acid substitution (S79L) that causes production of a truncated glycan at the surface of the MCP [41]. NC64A transformation was assessed by monitoring transient *β*-glucuronidase (GUS) and *gfp* expression 2 days after algal and bacterial co-incubation.

From among a few hundred colonies containing putative transformants that appeared within 20 days on selection media (containing 20 mg/L hygromycin and 500 mg/L cefotaxime) (Fig 5C) a total of eight hygromycin-resistant single colonies were randomly selected and grown in liquid media (containing 500 mg/L cefotaxime but lacking hygromycin) before the DNA was extracted and used in PCR analysis. Amplification with EPA1-*a064r* domain 1 (D1) gene-specific primers successfully detected the 633 bp a*064r* gene fragment from seven putative transgenic lines (Fig 5A), which represents 87.5% of the total number of screened colonies. To rule out *Agrobacterium* contamination, amplification with Kan^R gene-specific primers only produced the expected 795 bp Kan^R gene fragments in LBA4404 (positive control) but not in the seven putative NC64A transgenic lines (Fig 5B). The presence and absence of these gene-specific fragments in seven putative transgenic lines with no amplification product detected in non-infected wild-type NC64A cells indicated the successful transfer of both the hygromycin marker and the GFP-GUS reporter gene as a single T-DNA unit flanked by the left and right borders. The identity of the PCR amplicons derived from transgenic NC64A colonies (Fig 5C) were further confirmed to be positive by DNA sequencing where sequence alignment showed 99 and 98% identity to the *hpt* and *gfp-gusA* genes in pCAMBIA1303 respectively (Gene Bank accession no. AF234300).

Fig 5. PCR analysis of putative NC64A transformants transformed with recombinant pCAMBIA1303 *034r* domain 1.

(A) Amplification of the 633 bp fragment of the *a064r* domain 1 gene fragment from four LBA4404 colonies post-electroporation, and eight NC64A colonies after *Agrobacterium*-mediated transformation. (B) Amplification of the Kan^R gene from LBA4404 (positive control) but not in the seven putative NC64A transgenic lines. (C) Transgenic NC64A colonies on selective media (containing 20 mg/L hygromycin and 500 mg/L cefotaxime).

Confocal fluorescent microscopy of NC64A cells expressing the *gfp* gene. Visualization of GFP using a fluorescent microscope confirmed weak expression of the *gfp* gene in cells early after transformation (Fig 6). However, the failure of DNA isolated from many hygromycin-resistant colonies to produce DNA amplification products from either of two PCR primer pairs indicates the potential for 'escapes' through antibiotic selection. The seven transgenic NC64A lines that were previously confirmed positive for EPA1-*a064r* D1 by PCR could not be maintained or subcultured on hygromycin-supplemented media. No GUS-positive cells were detected in transgenic lines post-selection with hygromycin, although PCR amplification confirmed the presence of the *gfp-gusA* gene (data not shown). This indicates the possibility of silencing of the *gfp-gusA* gene and other transgenes in transgenic lines.

Fig 6. Green fluorescence of NC64A cells transformed by *A. tumefaciens* containing the pCAMBIA1304 binary vector carrying the gfp–gusA reporter gene.

Confocal fluorescent microscopy of live NC64A cells transformed with *A. tumefaciens* containing pCAMBIA1304 as imaged through green, red, and GFP channels (from left to right). The GFP

468 channel displays fluorescence due to expression of the green fluorescent protein. Bar represents
469 approximately 5 μm.
470
471 **Discussion**
472 Reverse genetic manipulation of NC64A genomes is currently not possible and as a result
473 directed modification of chloroviruses has yet to be achieved. We attempted to produce
474 recombinant chloroviruses for many years by placing plasmids containing a virus gene (including
475 the glycosyltransferase described in this manuscript) into host alga cells and then infecting with a
476 chlorovirus and screening for recombinant nascent viruses as is done for making recombinant
477 vaccinia viruses [42]. Genetic recombination is known to occur in the chloroviruses [43, 44].
478 Although many vectors were constructed and various ways to introduce the plasmids into the host
479 alga were tried, recombinant viruses were never obtained. The lack of success in some of the
480 earliest experiments was initially thought to be due to the fact that the chloroviruses produce and
481 package virus-encoded DNA restriction endonucleases in their virions. These restriction enzymes
482 are involved in digesting the host DNA shortly after virus infection. However, despite the fact that
483 many of the more recent attempts were conducted with virus CA-4B, which does not appear to
484 code for a restriction endonuclease, success was not obtained.
485 Consequently, with the advent of the CRISPR/Cas9 system there was hope that such
486 problems might be overcome. In the research reported here, we attempted to develop a
487 transformation system to generate stable site-directed chlorovirus mutants by targeting a
488 chlorovirus glycosyltransferase-gene that conveniently provided a distinguishable and selectable
489 glycan phenotype. Despite our efforts to improve methods to penetrate host NC64A cells and

490 modify CA-4A viral DNA, we were unable to produce a reliable transformation system that
491 supported the genetic modification of chloroviruses.

492 The most promising results in the present study was the recovery of two CA-4B mutants
493 that harbored hallmark, sequence-verified, Cas9/sgRNA RNP-directed indels. However, despite
494 exhaustive numbers of trails and alternative enzyme combinations, we were unable to duplicate
495 these results. It is unlikely that detection of the mutant virus DNA was due to erroneous sequencing
496 given that all samples were sequenced in triplicate with consistent readings. One possible
497 explanation for obtaining the RNP-mediated NHEJ event in CA-4A viral DNA is that macerozyme
498 on a rare occasion successfully eroded the alga cell wall, promoting cell permeability, allowing
499 the entrance of the RNP-targeting cargo. Despite targeting cell-wall polymers, macerozyme did
500 not compromise all the host receptors sufficiently to prevent virus attachment as evidenced by
501 routinely productive virus infections in macerozyme-treated cells. The host receptor, which is
502 likely a carbohydrate, is uniformly present over the entire surface of the alga [45]. Once inside the
503 infected cell, deposited viral DNA could be recognized by the gene-targeting RNPs. The lack of
504 success of the SiC and electroporation procedures in producing mutant viruses is somewhat
505 surprising given evidence of RNP delivery inside NC64A was greater using these delivery
506 techniques compared to macerozyme-treated cells (Fig 3). Perhaps the fluorescently labeled cells,
507 although in greater quantity, were either nonviable or compromised in health that prevented the
508 desired viral DNA editing.

509 In one study [46], 14 different strains of *Chlorella* were challenged with cell wall-
510 degrading enzymes and no two strains had the same pattern of inhibition. This large range of
511 sensitivity to various enzymatic activities illustrates the wide diversity of cell wall composition
512 amongst the *Chlorella* alga species. Our results show that in methodologies or processes using

intact algal cells or residual algal biomass, enzymatic treatment can have large impacts on the permeability of the algal cell walls and may be useful in optimization of various processes.

FACS analysis of SiC whiskers-treated and electroporated NC64A cells showed ~12% and ~20% cell fluorescence, respectively, suggesting positive delivery of the RNPs inside the alga cells. We speculate that cell wall perforations either induced by mechanical force or voltage allowed Cas9/sgRNA RNP complexes brief access to the inside of the cell. In theory, once viral DNA is deposited inside the alga cell, the naked nucleic acid is available for Cas9/sgRNA binding and subsequent *034r* gene cleavage. The edited viral DNA would be subsequently replicated and packaged. Following antibody selection, we expected to recover an NHEJ indel event in CA-4B at the RNP-designated target site, however, with just two exceptions, we were unsuccessful. Although putatively mutant virus plaques were recovered by our screening techniques, sequencing results confirmed that in the vast majority of recovered viruses the wildtype *034r* gene was intact. Given these sequencing results, the plaques produced were likely caused by escape viruses that evaded wildtype antibody binding.

We also briefly examined the effect of parameters involving cell-penetrating peptides, such as the peptide/RNP ratio and serum addition, on peptide-mediated transfection. Properties of peptides (DNA binding stability and condensation capacity) and of peptide/RNP complexes (size and surface charge) were investigated because these are known to vary as a function of the peptide/RNP ratio. Following antibody selection, we expected to recover an NHEJ indel event in CA-4B at the Cas9/sgRNA RNP-designated target site. However, we were unsuccessful. Although virus plaques were recovered, sequencing results confirmed the wildtype *034r* gene was intact. Given these sequencing results, these plaques, again, were likely caused by escape viruses that evaded wildtype antibody binding.

Finally, our results from *Agrobacterium*-mediated transformation of NC64A demonstrated its potential for being a reasonably promising transformational system to further pursue and optimize. Here, seven PCR-positive transformants were obtained, and the presence of the viral gene fragment suggests that the T-DNA was integrated into the NC64A genome; however, more extensive studies are needed to elucidate possible factors and mechanisms contributing to the loss of the *gfp-gusA* expression and hygromycin resistance. Approaches to prevent or minimize gene silencing might prove useful in maintaining the expression of newly introduced genes. Other vector systems and/or promoters might also need to be tested with NC64A to develop a better transformation system. Nonetheless, the finding opens the possibility of further genetic manipulation of this commercially important microalga with other genes of interest.

Broadly speaking, genetic modification of chloroviruses would provide functional insight into the unusual chlorovirus encoded proteins mentioned previously such as hyaluronan synthase, potassium ion channel protein, five polyamine biosynthetic enzymes, and as addressed here, glycosyltransferases. Furthermore, adoption of a reverse genetics system would also allow the exploration of formerly characterized proteins having potential scientific and economic benefit. For example, chlorovirus genes encode commercially important enzymes such as DNA restriction endonucleases and contain elements for genetically engineering other organisms. Examples include viral promoter elements that function well in both monocot and dicot higher plants, as well as bacteria [47, 48], and a translational enhancer element that functions in *Arabidopsis* [49]. Chloroviruses have some of the smallest, most primitive forms of highly complex proteins that exist in higher organisms serving as a simplified fossil template to study biochemical models for mechanistic and structural studies [50]. Therefore, the development of a successful and reproducible procedure for achieving genetic modification of chloroviruses would accelerate the

559 exploration of microalgae and their viruses for a broader range of scientific investigations and
560 biotechnological applications. Such an achievement would be a major step forward.
561
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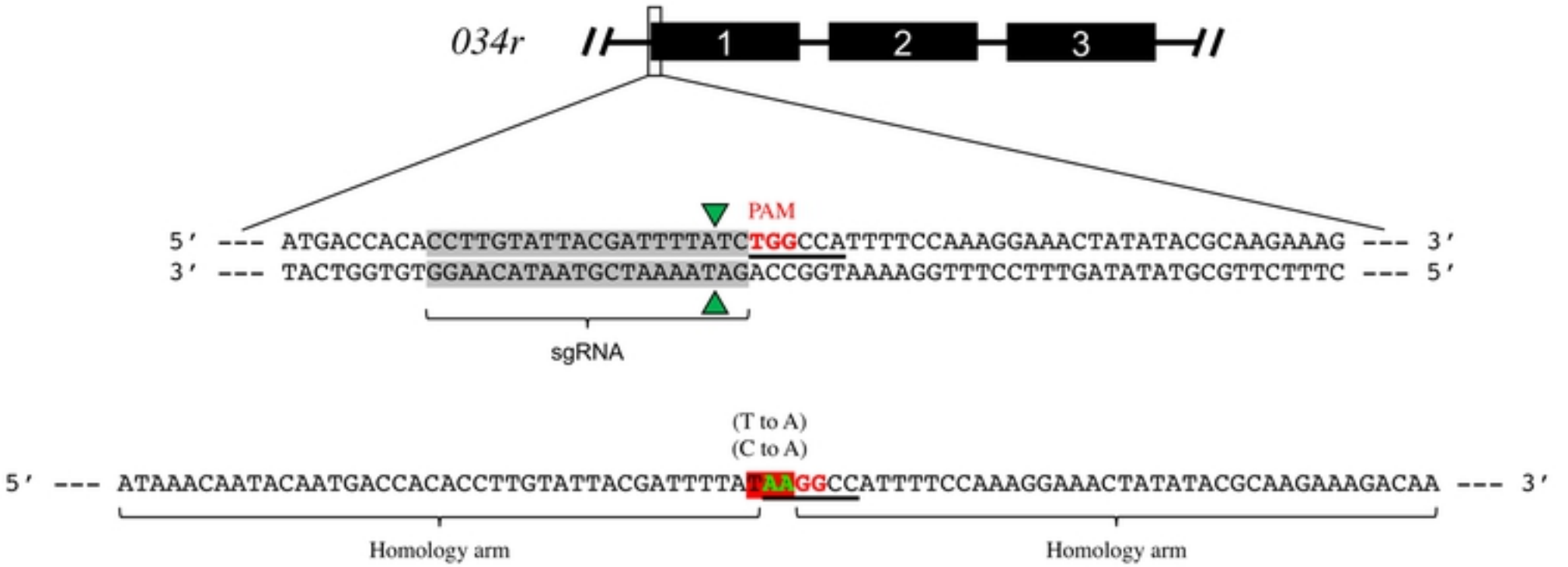
034r
1
2
3
PAM
5′ --- ATGACCACACCTTGTATTACGATTTTATCTGGCCATTTTCCAAAGGAAACTATATACGCAAGAAAG --- 3′
3′ --- TACTGGTGTGGAACATAATGCTAAAATAGACCGGTAAAAGGTTTCCTTTGATATATGCGTTCTTTC --- 5′
sgRNA
(T to A)
(C to A)
5′ --- ATAAACAATACAATGACCACACCTTGTATTACGATTTTATAAGGCCATTTTCCAAAGGAAACTATATACGCAAGAAAGACAA --- 3′
Homology arm
Homology arm

Fig 1

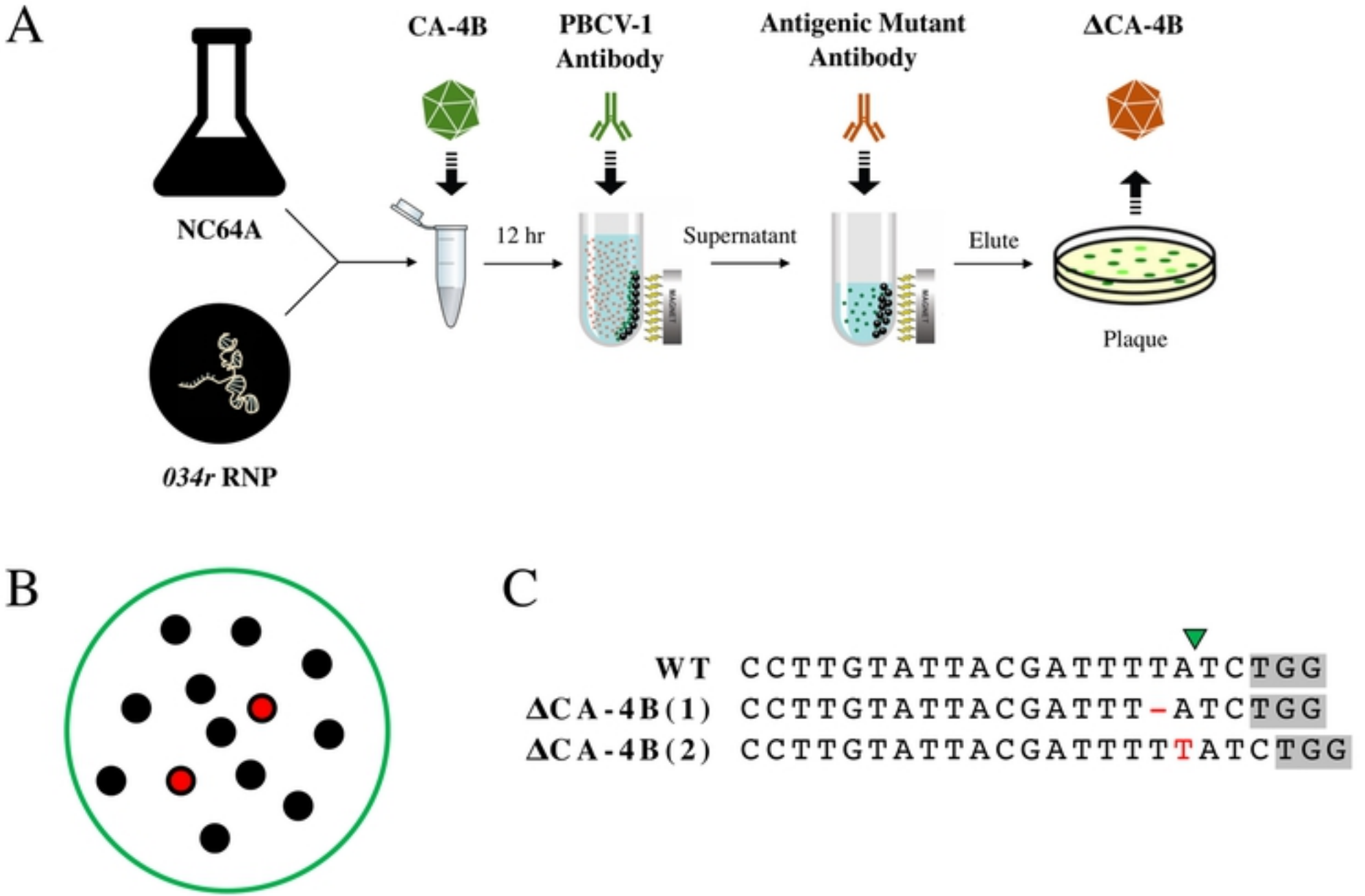
A
CA-4B
PBCV-1 Antibody
Antigenic Mutant Antibody
ΔCA-4B
NC64A
034r RNP
12 hr
Supernatant
Elute
Plaque
B
C
WT CCTTGTATTACGATTTTATCTGG
ΔCA-4B(1) CCTTGTATTACGATTT-ATCTGG
ΔCA-4B(2) CCTTGTATTACGATTTTTATCTGG

Fig 2

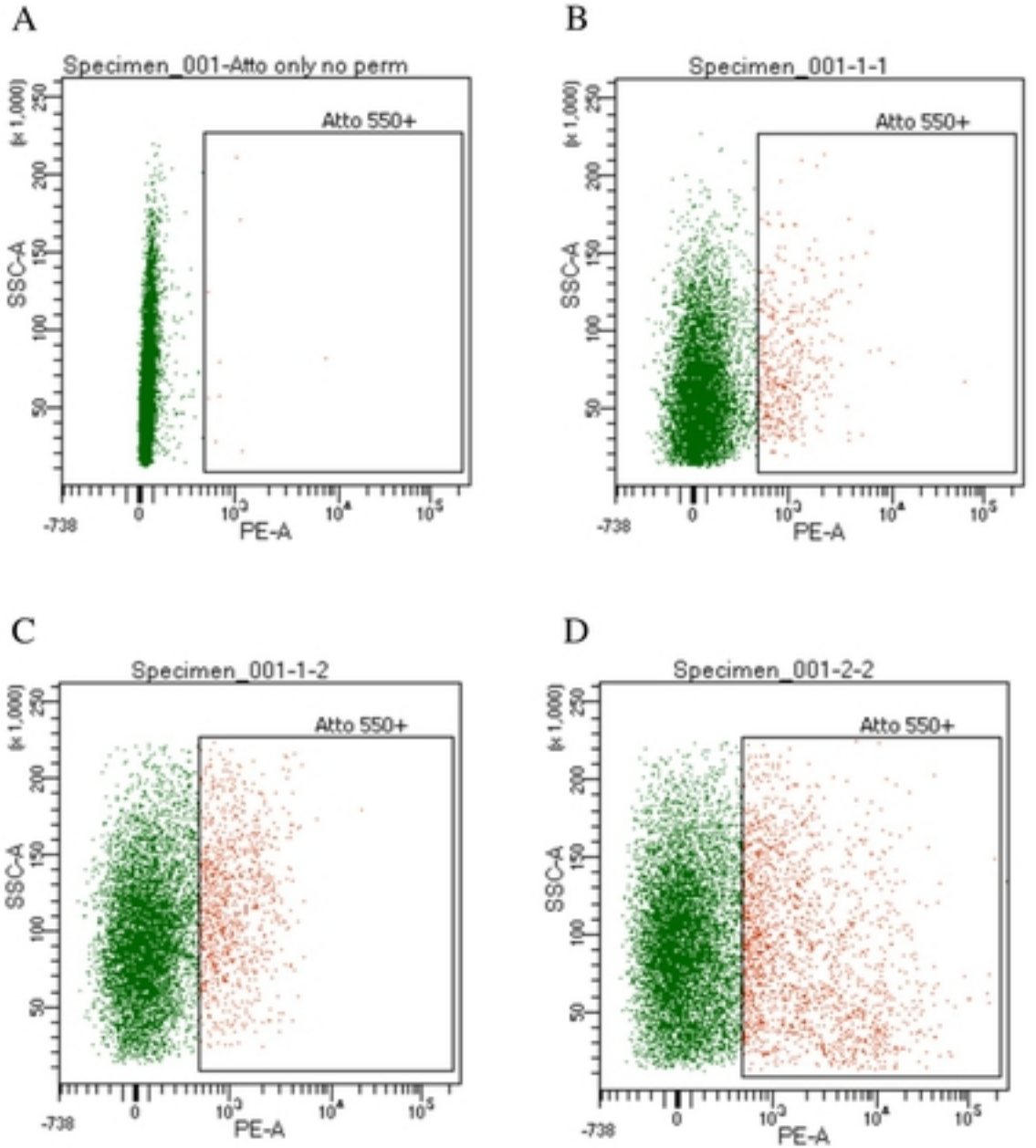
A
Specimen_001-Atto only no perm
Atto 550+
SSC-A
(x 1,000)
PE-A
B
Specimen_001-1-1
Atto 550+
C
Specimen_001-1-2
Atto 550+
D
Specimen_001-2-2
Atto 550+

Fig 3

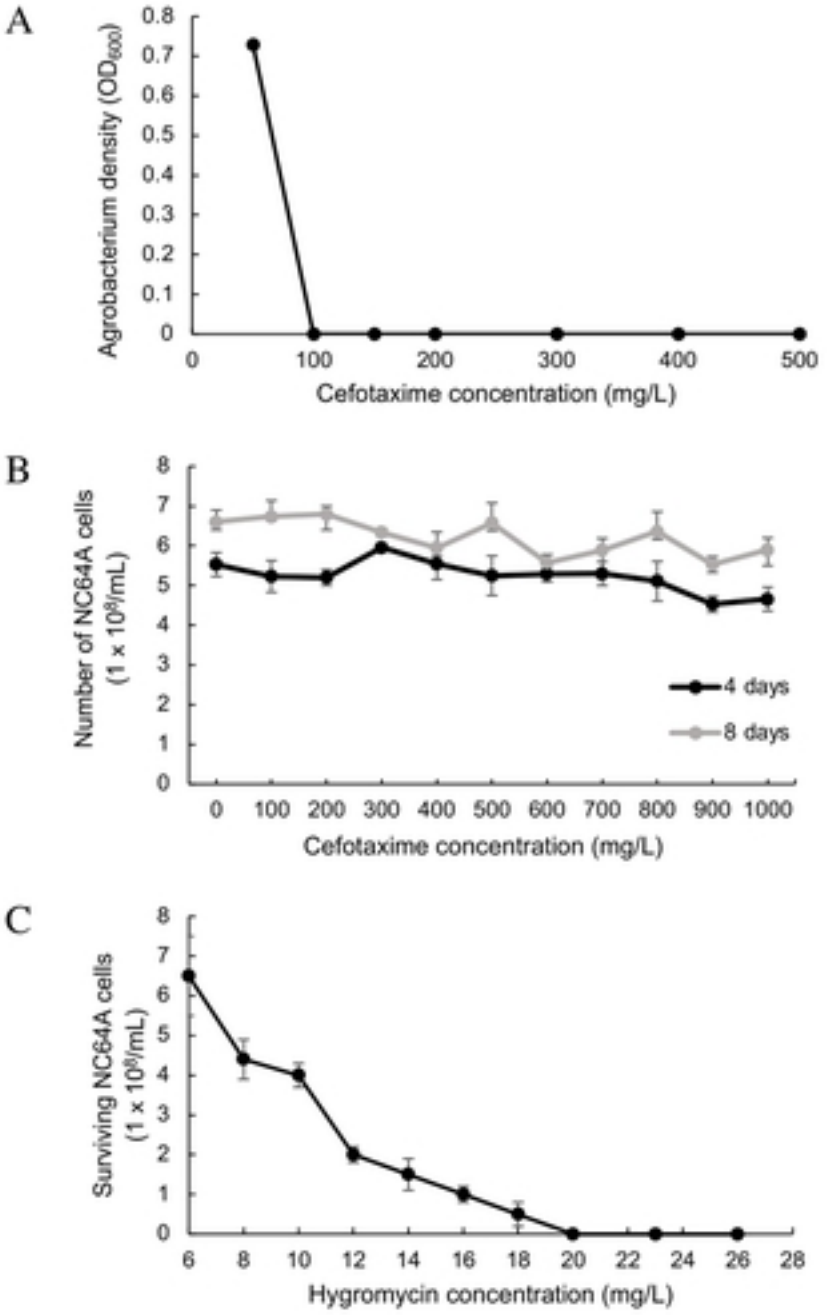
A
Agrobacterium density (OD_{600})
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
Cefotaxime concentration (mg/L)
B
Number of NC64A cells
(1 x 10^8/mL)
8
7
6
5
4
3
2
1
0
4 days
8 days
0 100 200 300 400 500 600 700 800 900 1000
Cefotaxime concentration (mg/L)
C
Surviving NC64A cells
(1 x 10^8/mL)
8
7
6
5
4
3
2
1
0
6 8 10 12 14 16 18 20 22 24 26 28
Hygromycin concentration (mg/L)

Fig 4

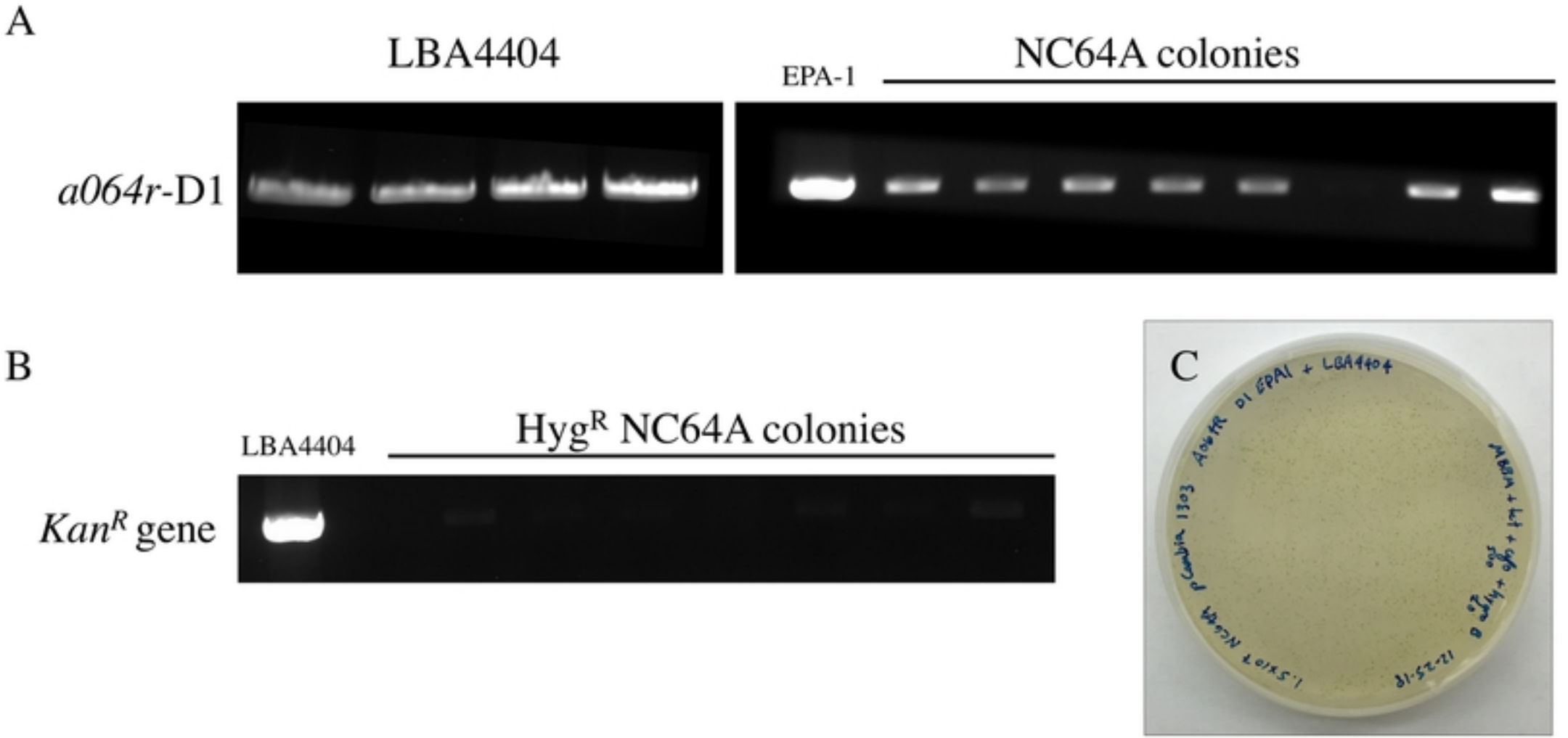
A
LBA4404
EPA-1
NC64A colonies
a064r-D1
B
LBA4404
Hyg^R NC64A colonies
Kan^R gene
C

Fig 5

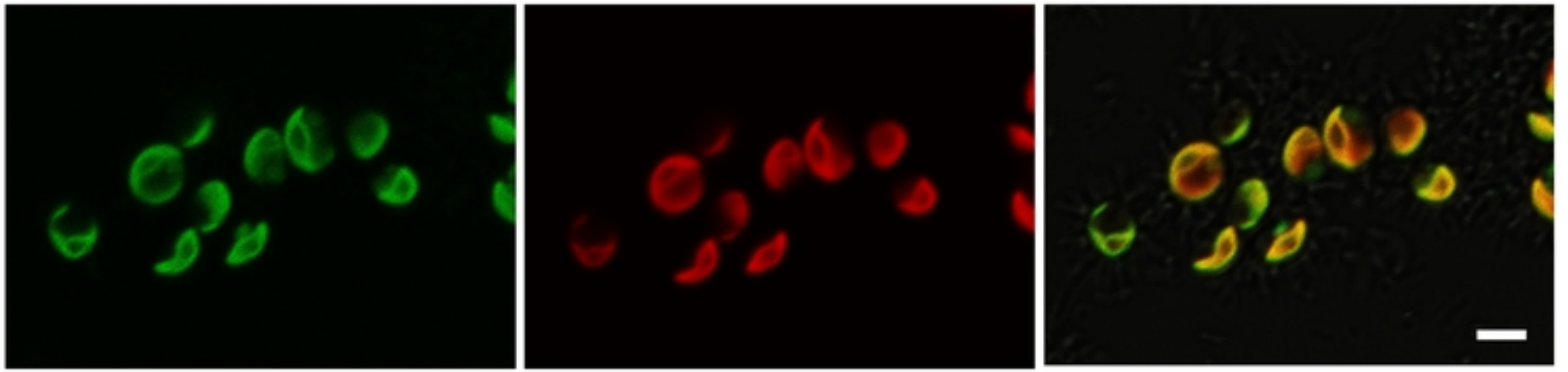

Fig 6