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Title: CRISPR-Cas9 Genome Editing in *Mesorhizobium ciceri* Through *nodC* Disruption for Chickpea Symbiosis Studies

Authors and Affiliations:

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera? **Yes**

SCOPE shots: 2.2.1, 2.2.2, 2.8.1

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No, we need to display only the values appearing on the screen.**

SCREEN shots: 2.4.1, 2.4.3, 2.8.2, 2.8.3, 3.13.1, 3.13.2, 3.13.3

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 22

Number of Shots: 54

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Nandita Pasari:** Our research employs a broad-host-range CRISPR-Cas9 system, enabling efficient *Mesorhizobium ciceri* genome editing for functional genomics studies.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Nandita Pasari:** This protocol details a robust CRISPR-mediated genome editing approach for *Mesorhizobium ciceri*, bypassing labor-intensive, conventional suicide-plasmid methods.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Nandita Pasari:** This protocol will enables researchers to generate gene disruptions and study gene functions in *Mesorhizobium*.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Nandita Pasari:** The main challenge in this protocol is isolating individual transformed colonies.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. **Nandita Pasari:** The nod-deleted M. ciceri strain generated using this protocol can be used as a control to identify genes associated with root nodulation in the wild-type strain.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Biparental Conjugation of gRNA-HDR Plasmid Constructs into *M. ciceri* for *nodC* Disruption

Demonstrator: Nandita Pasari

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

- 2.1. To begin, transform the donor *Escherichia coli* S17-1 λ pir (*E-KOH-Iye S-seventeen-one lambda-peer*) cells with the target guide RNA, or gRNA (*G-R-N-A*) and homology-directed repair, or HDR (*H-D-R*) containing plasmid constructs [1]. Select the transformants on LB agar containing 50 micrograms per milliliter of neomycin [2].
 - 2.1.1. WIDE: Talent transforming *E. coli* S17-1 λ pir cells with the pCasPP-gRNA1-HDR or pCasPP-gRNA2-HDR constructs. **TXT: Target plasmids: pCasPP-gRNA1-HDR or pCasPP-gRNA2-HDR**
 - 2.1.2. Talent selecting the transformants on LB agar containing neomycin.
- 2.2. Screen the transformants for GFP (*G-F-P*) fluorescence using excitation at approximately 488 nanometers and emission detection at approximately 510 to 520 nanometers [1]. Classify the colonies as GFP-positive if they exhibit uniform, distinct green fluorescence that is absent from non-transformed *Escherichia coli* control colonies under identical microscope settings [2]. Prepare glycerol stocks from the confirmed GFP-positive donor clones [3].
 - 2.2.1. SCOPE: Transformant colonies being screened for green fluorescent protein fluorescence.
 - 2.2.2. SCOPE: Green fluorescent protein-positive colonies visible with uniform, distinct green fluorescence compared with non-transformed control colonies.
 - 2.2.3. Talent preparing glycerol stocks from confirmed green fluorescent protein-positive donor clones.
- 2.3. To prepare the donor starter cultures, inoculate a single colony of *Escherichia coli* into 10 milliliters of LB (*L-B*) broth containing 50 micrograms per milliliter of neomycin [1]. Incubate overnight at 37 degrees Celsius with shaking at 180 rpm [2]. Simultaneously, inoculate the recipient *Mesorhizobium ciceri*, strain into 10 milliliters of yeast mannitol broth, or YMB (*Y-M-B*), supplemented with 100 micrograms per milliliter carbenicillin [3]. Incubate the recipient cultures at 28 degrees Celsius with shaking at 180 rpm for 24 to 30 hours [4].
 - 2.3.1. Talent inoculating the donor *E. coli* S17-1 λ pir into 10 mL LB broth.

- 2.3.2. Talent placing the donor starter culture in the shaker incubator.
- 2.3.3. Talent inoculating the recipient *M. ciceri* starter culture into YMB.
- 2.3.4. Talent placing the recipient culture on the shaker incubator.
- 2.4. ~~Grow the starter cultures to an optical density or OD (O-D) of 0.3 to 0.4 at 600 nanometers, corresponding to an estimated cell density of approximately 100 million colony forming units per milliliter [1].~~ Prepare donor and recipient subcultures by diluting the respective starter stocks to 1 in 100 into fresh selective media [2]. Grow them to the required exponential growth phase, as indicated by an OD600 (O-D-six-hundred) of 0.4 to 0.8 [3]. NOTE: The VO is edited for the deleted shot
- 2.4.1. ~~SCREEN: Show the OD values of the starter cultures of both *E. coli* and *M. ciceri*.~~ NOTE: Not filmed
- 2.4.2. Talent preparing donor and recipient subcultures.
- 2.4.3. SCREEN: Show the OD values of subcultures reaching 0.4 to 0.8
- 2.5. For membrane-filter biparental mating, spot four 50 to 100 microliter aliquots of a 1 to 3 ratio of donor-to-recipient culture mixture, resuspended in 10 millimolar magnesium chloride, onto a sterile PVDF membrane filter placed on a non-selective yeast mannitol agar plate [1-TXT]. Space the spots adequately to prevent mixing [2], then incubate upright at 28 degrees Celsius for 5 to 7 days [3].
 - 2.5.1. Talent spotting the mating suspension onto a sterile polyvinylidene fluoride membrane filter. TXT: Filter size: 0.22 µm; Diameter: 47 mm
 - 2.5.2. Talent spacing the aliquots on the membrane filter.
 - 2.5.3. Talent carefully placing the plate in the incubator.
- 2.6. To recover the transconjugants, initially perform dual-antibiotic selection against neomycin and carbenicillin [1]. Next, passage the transconjugants repeatedly on carbenicillin-only medium to eliminate residual plasmids [2].
 - 2.6.1. Talent recovering transconjugants for dual-antibiotic selection.
 - 2.6.2. Talent passaging transconjugants on carbenicillin-only medium.
- 2.7. Confirm plasmid curing by the failure of colonies to grow on neomycin-containing medium, simultaneously plating the transconjugants on carbenicillin-containing media [1], and by diagnostic PCR (P-C-R) demonstrating the absence of the pCasPP (P-kass-P-P) backbone while retaining the chromosomally integrated HDR cassette [2].
 - 2.7.1. Talent checking colonies on neomycin-containing medium and plating on carbenicillin-containing media
 - 2.7.2. Talent performing diagnostic polymerase chain reaction.
- 2.8. Screen purified transconjugant colonies for GFP fluorescence and compare them with wild-type *Mesorhizobium ciceri* controls [1]. Select fluorescent colonies as putative

HDR-positive strains with uniform, distinct green fluorescence [2], followed by confirmation with diagnostic PCR before downstream validation [3].

- 2.8.1. SCOPE: Purified transconjugants being compared with wild-type *M. ciceri* controls for green fluorescent protein fluorescence.
- 2.8.2. SCREEN: Show the selection of fluorescent colonies as putative HDR-positive strains.
- 2.8.3. SCREEN: Show the results of diagnostic PCR and confirmation of GFP-positive colonies.
- 2.9. Store the confirmed colonies on yeast mannitol agar at 4 degrees Celsius for short-term maintenance [1] or as glycerol stocks containing 20 percent to 25 percent glycerol at minus 80 degrees Celsius for long-term preservation [2].
 - 2.9.1. Talent storing confirmed colonies on yeast mannitol agar.
 - 2.9.2. Talent preparing glycerol stocks from confirmed colonies for long-term preservation.

3. Functional Validation of *Mesorhizobium ciceri* nodC Mutants

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

- 3.1. To assess the nodulation potential after verifying the GFP integration at the nodC locus, inoculate single colonies of the wild-type and mutant strains into selective YMB containing 100 micrograms per milliliter carbenicillin [1]. Incubate the cultures at 28 degrees Celsius with shaking at 180 rpm for 30 to 36 hours [2].
 - 3.1.1. Talent inoculating single colonies into selective yeast mannitol broth containing carbenicillin.
 - 3.1.2. Talent placing the culture flasks in the shaking incubator.
- 3.2. Dilute the cultures at 1 in 100 ratios into fresh selective YMB [1]. Use the ultraviolet-visible spectrophotometer to measure the OD600 after blanking the instrument with uninoculated YMB [2]. Continue incubation until the cultures reach an OD600 of 0.3 to 0.4, corresponding to approximately 100 million colony-forming units per milliliter [3].
 - 3.2.1. Talent diluting the cultures into fresh selective yeast mannitol broth.
 - 3.2.2. Talent measuring the optical density at 600 nanometers with a ultraviolet-visible spectrophotometer.
 - 3.2.3. Talent placing the cultures back for incubation.
- ~~3.3. Use 3 milliliters of the standardized bacterial suspension for inoculation of each plant [1].~~
 - ~~3.3.1. Talent dispensing 3 milliliters of the standardized bacterial suspension into a container for plant inoculation.~~ **NOTE: This step is not required, hence removed**

- 3.4. For chickpea plant infection assay, obtain seeds of a specific chickpea plant variety [1-TXT]. Wash the seeds thoroughly with running tap water, then with Milli-Q purified water [2].
 - 3.4.1. Talent obtaining the *Cicer arietinum* cultivar BDG25618 seeds. **TXT: Plant variety: *Cicer arietinum* cultivar BDG25618**
 - 3.4.2. Talent washing the seeds with running tap water followed by Milli-Q purified water.
- 3.5. Surface-sterilize the seeds in 1 percent sodium hypochlorite for 5 minutes with agitation, followed by 5 times rinsing with sterile purified water [1]. Then, treat the seeds with 0.1 percent Bavistin fungicide containing 50 percent carbendazim wettable powder for 2 to 3 hours at room temperature [2]. Wash the treated seeds 2 times with sterile purified water [3].
 - 3.5.1. Talent surface-sterilizing the seeds.
 - 3.5.2. Talent treating the seeds with Bavistin fungicide.
 - 3.5.3. Talent washing the treated seeds with sterile purified water.
- 3.6. Place the seeds on moist sterile filter paper in sterile Petri dishes measuring 110 millimeters by 40 millimeters [1]. Seal the dishes with Parafilm and incubate them in the dark at approximately 28 degrees Celsius for 3 days [2].
 - 3.6.1. Talent placing the seeds on moist sterile filter paper in sterile Petri dishes.
 - 3.6.2. Talent sealing the Petri dishes with Parafilm and placing them for incubation.
- 3.7. Transfer the germinated seedlings individually into sterile sand, autoclaved at 121 degrees Celsius and 15 pounds per square inch for 20 minutes before use [1].
 - 3.7.1. Talent transferring the germinated seedlings into sterile sand.
- 3.8. Dissolve the components to prepare the nitrogen-free McKnight's nutrient solution [1-TXT]. Grow the seedlings in 10-centimeter-diameter pots containing sterile sand, maintaining 4 plants per pot and irrigating each pot twice weekly with 20 milliliters of the nitrogen-free McKnight's nutrient solution [2].
 - 3.8.1. Talent preparing the nitrogen-free McKnight's nutrient solution. **TXT: Refer to text for media composition**
 - 3.8.2. Talent transferring the seedlings into pots containing sterile sand.
- 3.9. Maintain the plants [1] under controlled growth conditions [2]. Grow the seedlings for 6 to 7 days before inoculation with mutant bacteria [3].
 - 3.9.1. Talent placing the plants under controlled growth conditions.
 - 3.9.2. TEXT ON A PLAIN BACKGROUND

Controlled Growth Conditions

Day temperature	22°C ± 2°C
Night temperature	18°C ± 2°C
Relative Humidity	55%–60%
Photoperiod	16 h light/8 h dark
Light Intensity	200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

3.9.3. Shot of seedlings growing before inoculation.

3.10. Apply 3 milliliters of the bacterial suspension directly to each seedling [1]. For the mock-treated control seedlings, apply 3 milliliters of sterile YMB [2]. Maintain 16 biological replicate plants for each treatment group [3].

3.10.1. Talent applying the bacterial suspension to each seedling.

3.10.2. Talent applying sterile yeast mannitol broth to the mock-treated control seedlings.

3.10.3. Shot of the treatment groups containing the biological replicate plants.

3.11. Harvest the roots and shoots 20 days after inoculation [1]. Count the nodules manually [2] and record the fresh biomass immediately after harvest [3].

3.11.1. Talent harvesting the roots and shoots.

3.11.2. Talent counting the nodules manually.

3.11.3. Talent recording the fresh biomass.

3.12. Dry the tissues at 65 degrees Celsius until a constant weight is achieved [1]. After verifying the constant weight using 2 consecutive 24-hour measurements that differ by less than 0.01 grams, record the dry biomass [3].

3.12.1. Talent placing the tissue samples for drying.

3.12.2. Talent recording the dry biomass.

3.13. Express the results as the mean plus or minus the standard error [1]. Perform a one-way analysis of variance followed by Tukey's multiple-comparison test using **GraphPad Prism** version 10.0 [2]. Consider differences to be statistically significant [3].

3.13.1. SCREEN: 3.13.1.mp4: 00:15-00:19, 01:55-02:00.

3.13.2. SCREEN: 3.13.2.mp4: 00:18-00:21, 00:27-00:33, 01:08-01:18, 01:24-01:26

3.13.3. SCREEN: 3.13.3.mp4: 00:47-00:55

Results

4. Results

- 4.1. Following biparental mating, transconjugant colonies were initially identified by GFP fluorescence screening [1]. More than 80 percent of recovered colonies displayed GFP fluorescence with gRNA2 (*G-R-N-A-2*) [2], and more than 65 percent of colonies exhibited fluorescence following the introduction of gRNA1 (*G-R-N-A-1*) [3].
 - 4.1.1. LAB MEDIA: Revised Figure 3C Microscope Image. *Video Editor: Please emphasize both the left panels showing the green fluorescence.*
 - 4.1.2. LAB MEDIA: Revised Figure 3C Graph. *Video Editor: Please emphasize the Y-axis value corresponding to the bar labelled gRNA2.*
 - 4.1.3. LAB MEDIA: Revised Figure 3C Graph. *Video Editor: Please emphasize the Y-axis value corresponding to the bar labelled gRNA1.*
- 4.2. Validation of GFP cassette integration by PCR amplification of the genomic region spanning *nodB* (*nod-B*) and *nodC* (*nod-C*) produced a 1511 basepair fragment in the edited clones, consistent with integration [1], whereas the wild-type strain produced a 430 basepair amplicon [2]. Further, PCR amplification using GFP-specific and *nodC*-flanking primers generated a distinct 997 basepair fragment in the edited clones [3], while no amplification was detected in the wild-type control, supporting locus-specific insertion [4].
 - 4.2.1. LAB MEDIA: Figure 4A (Left side gel image). *Video Editor: Please emphasize the 4 bands corresponding to the 1511 bp label along with the label.*
 - 4.2.2. LAB MEDIA: Figure 4A (Left side gel image). *Video Editor: Please emphasize the single band corresponding to the 430 bp label along with the label.*
 - 4.2.3. LAB MEDIA: Figure 4A (Right side gel image). *Video Editor: Please emphasize the 4 bands corresponding to the 997 bp label along with the label.*
 - 4.2.4. LAB MEDIA: Figure 4A (Right side gel image). *Video Editor: Please emphasize the absence of 997bp band in the final lane labelled WT at the top.*
- 4.3. Functional validation of mutant *Mesorhizobium ciceri* by chickpea infection assay shows a marked reduction or absence of nodulation in plants inoculated with mutant bacteria [1], whereas plants inoculated with wild-type bacteria showed formation of visible root nodules [2]. Wild-type-inoculated plants exhibited higher nodule numbers and improved growth-associated parameters [3], compared with mutant-inoculated [4] and uninoculated control plants [5].
 - 4.3.1. LAB MEDIA: Figure 5A. *Video Editor: Please emphasize the plant labelled “ΔnodC M. Ciceri”*

- 4.3.2. LAB MEDIA: Figure 5A. *Video Editor: Please emphasize the plant labelled “M. Ciceri”*
- 4.3.3. LAB MEDIA: Figure 5B. *Video Editor: Please emphasize the height of bars in all graphs with X-axis label “M.ciceri”*
- 4.3.4. LAB MEDIA: Figure 5B. *Video Editor: Please emphasize the height of bars in all graphs with X-axis label “ Δ nodC M. Ciceri”*
- 4.3.5. LAB MEDIA: Figure 5B. *Video Editor: Please emphasize the height of bars in all graphs with X-axis label “Uninoculated”*

- 1. Escherichia coli
Pronunciation link: <https://www.merriam-webster.com/dictionary/E.%20coli>
IPA: / ɛʃəˈrɪkiə ˈkoʊlaɪ/
Phonetic Spelling: esh·uh·RIK·ee·uh KOH·lye
- 2. Neomycin
Pronunciation link: <https://www.merriam-webster.com/dictionary/neomycin>
IPA: / ˌniːəˈmaɪsɪn/
Phonetic Spelling: nee·uh·MY·sin
- 3. Mesorhizobium ciceri
Pronunciation link: No confirmed link found
IPA: / ˌmɛzoʊraɪˈzoʊbiəm ˈsɪsərəɪ/
Phonetic Spelling: mez·oh·rye·ZOH·bee·um SIS·uh·rye
- 4. Mannitol
Pronunciation link: <https://www.merriam-webster.com/dictionary/mannitol>
IPA: / ˈmæniˌtɔːl/
Phonetic Spelling: MAN·ih·tawl
- 5. Carbenicillin
Pronunciation link: <https://www.merriam-webster.com/medical/carbenicillin>
IPA: / ˌkɑːrˌbɛnɪˈsɪlɪn/
Phonetic Spelling: kar·ben·ih·SIL·in
- 6. Biparental
Pronunciation link: <https://www.merriam-webster.com/dictionary/biparental>
IPA: / ˌbaɪpəˈrentəl/
Phonetic Spelling: bye·puh·REN·tuhl
- 7. Polyvinylidene fluoride
Pronunciation link: No confirmed link found

- IPA: /ˌpɑːliˌvænəˈlɪdiːn ˈflʊərəɪd/
Phonetic Spelling: pol·ee·vy·nuh·LID·een FLOOR·yde
8. Transconjugants
Pronunciation link: No confirmed link found
IPA: /ˌtrænzˈkɑːndʒəɡənts/
Phonetic Spelling: tranz·KON·juh·guhnts
9. Chromosomally
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromosomal>
IPA: /ˌkroʊməˈsoʊməli/
Phonetic Spelling: kroh·muh·SOH·muh·lee
10. Nodulation
Pronunciation link: <https://www.merriam-webster.com/dictionary/nodulation>
IPA: /ˌnɑːdʒəˈleɪʃən/
Phonetic Spelling: noj·uh·LAY·shuhn
11. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /ˌspektrəʊfəˈtɑːmɪtər/
Phonetic Spelling: spek·troh·fuh·TOM·uh·ter
12. Cicer arietinum
Pronunciation link: No confirmed link found
IPA: /ˈsɪsər ˈæriˈetɪnəm/
Phonetic Spelling: SIS·er air·ee·ET·ih·num
13. Sodium hypochlorite
Pronunciation link: <https://www.merriam-webster.com/dictionary/hypochlorite>
IPA: /ˈsoʊdiəm ˌhaɪpəˈklɔːraɪt/
Phonetic Spelling: SOH·dee·um hy·puh·KLOR·yte
14. Carbendazim
Pronunciation link: No confirmed link found
IPA: /kɑːrˈbendəzɪm/
Phonetic Spelling: kar·BEN·duh·zim
15. Inoculation
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculation>
IPA: /ɪˌnɑːkjəˈleɪʃən/
Phonetic Spelling: ih·nok·yuh·LAY·shuhn
16. Amplicon
Pronunciation link: <https://www.merriam-webster.com/dictionary/amplicon>
IPA: /ˈæmpləˌkɑːn/
Phonetic Spelling: AM·pluh·kon
17. Locus-specific
Pronunciation link: No confirmed link found
IPA: /ˈloʊkəs spəˈsɪfɪk/
Phonetic Spelling: LOH·kuhs spuh·SIF·ik
18. Uninoculated
Pronunciation link: No confirmed link found

IPA: /ˌʌnɪˈnɑːkjəˌleɪtɪd/

Phonetic Spelling: un·ih·NOK·yuh·lay·tid

19. Plasmid

Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>

IPA: /ˈplæzˌmɪd/

Phonetic Spelling: PLAZ·mid

20. Homology-directed repair

Pronunciation link: No confirmed link found

IPA: /həˈmɑːlədʒi dəˈreɪtɪd rɪˈpeɪr/

Phonetic Spelling: huh·MOL·uh·jee duh·REK·tid rih·PAIR

21. Fluorescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>

IPA: /flʊˈresəns/

Phonetic Spelling: floor·ES·uhns

22. Putative

Pronunciation link: <https://www.merriam-webster.com/dictionary/putative>

IPA: /ˈpjuːtətɪv/

Phonetic Spelling: PYOO·tuh·tiv

23. Autoclaved

Pronunciation link: <https://www.merriam-webster.com/dictionary/autoclave>

IPA: /ˈɔːtoʊklaɪvd/

Phonetic Spelling: AW·toh·klayvd

24. Photoperiod

Pronunciation link: <https://www.merriam-webster.com/dictionary/photoperiod>

IPA: /ˈfoʊtoʊˌpiəriəd/

Phonetic Spelling: FOH·toh·peer·ee·ud

25. Biomass

Pronunciation link: <https://www.merriam-webster.com/dictionary/biomass>

IPA: /ˈbaɪoʊˌmæs/

Phonetic Spelling: BY·oh·mass

26. Analysis of variance

Pronunciation link: No confirmed link found

IPA: /əˈnæləsɪs əv ˈveriəns/

Phonetic Spelling: uh·NAL·uh·sis uhv VAIR·ee·uhns