EMERALDS: A novel system for improved microbiome engineering and disease resistance in plants

Mohammad Ali Khan, Izaan Qamar Kiani, Fatima Noor Jawad, Syeda Rida Fatima, Namra Atif, Mohammad Hafi, Fatima Khan, Zainab Noor Jawad, Maha Zulfiqar Yousafi

Since we began to sow some 10,000 years ago, we have foraged to boost yield as a means of supporting the growing populace and tacking environmental obstacles. With global crop losses as high as 20 to 40 percent and disease intensity increasing 10% for every 1 degree Celsius temperature rise, there is a dire need to implement innovative schemes to boost crop productivity whilst ataining a sustainable model of agriculture. We are demonstrating a system whereby transcriptional regulators can be secreted into bacterial endophytes and pathogens via cell penetrating peptides to modulate their gene expression and thus impact disease progression and overall plant growth.
Keywords: Microbiome engineering, Climate change, Synthetic biology, Nicotiana.

Research Report

Beaconhouse Margalla Islamabad.

Contact for correspondence: igembmi@gmail.com

1/1/2025

BACKGROUND

With recent advancements in the study of plant microbiome, it has been proven that plants interact with the diverse microbiota colonizing the environment. Hence one implication of boosting productivity is tapping into the potential of the plant microbiome to craft microbial innovations that elevate productivity, with roots of such plant-microbial associations extending to over 200 years. Microbiota colonizing plant microbiome have been known to serve a variety of functions: from influencing nutrient uptake in the form of nitrogen-fixing bacteria in the root nodules and phosphate-solubilizing bacteria to inculcating disease resistance in the crops, inhibiting pathogenic microorganisms, reducing disease incidence. Microbiota amplify plant tolerance to abiotic stress factors such as drought and salinity. Some microbes release hormones such as auxins and cytokines, nurturing growth and water uptake. Microbes have also been known to improve the habitat of plants by enhancing soil structure and fertility, facilitating plant growth and productivity. Thus plants establish unique relations with the microbiota, crucial for their growth and resilience.

We thereby aim to exploit microbiome potential for harvesting food products that are safe and nutritious and to open new prospects of development in this field as a means of achieving sustainability.

Advances in microbial technologies appear promising but are shrouded by an array of challenges, including difficulties in establishing the introduced microbes, competition from native species and unpredictability of the environmental conditions. Despite showing potential, current methods often prove inconsistent in the real world demanding more practical and effective solutions.

Our project aims to tackle such hindrances by making use of d-Cas9 to facilitate such plant-microbial interactions with view to disease resistance and biological control, mitigating climatic stress and encouraging sustainability with reduced use of chemicals, heeding to reservations regarding their effects on the ecosystem over long term use.

dCas9- catalytically inactive Cas9 is engineered by inculcating specific point mutations to nullify its endonuclease activity. The D10A and H40A mutations deactivate the RuvC and HNH rendering Cas9 ineffective of gene editing whilst preserving its ability to bind to target sequences.

dCas9 can repress gene expression via steric hindrance by binding to the promoter or coding regions and can be fused to transcriptional activators for gene activation. With regard to gene activation one course of action is the use of scaffold RNAs which make use of the MS2 coat proteins fused to the SoxS activation domain, facilitating transcriptional activation at the desired genomic loci. This approach enable precise gene expression in bacteria, enabling both repression and activation.

Delivery methods come to mind: to facilitate the uptake of proteins by bacteria, we have decided to use the signal (1-3) beta endoglucanase signal peptide to efficiently secrete the protein to the apoplast, while fusing cell penetrating peptides to the dCas9 and SoxS to allow them to pass into bacterial cells. An mTurquoise2 fluorescent tag is attached to the C terminus of the dCas9 to visualize it's expression. We call this protein mod-dCas9. We chose the peptide TP10 for its transduction ability, tractability to fusion, and less tendency tio aggregate.

For our proof of concept, we plan in using dCas9-SoxS modified with Nicotiana benthamiana while using dCas9 alone to repress mCherry expression in Pseudomonas syringae infecting

Nicotiana leaves . In our research, we have made use of SoxS and dCas9 to activate mVenus in Bacillus subtilis endophytes.

We call it ***EMERALDS:*** Engineering Microbial Endophytes for Resilient Agriculture using dCas9.

METHODS

DNA sequences

DNA sequences used will be obtained from Twist Biosciences in whole.

Polymerase chain reaction (PCR)

AccuPrime™ Pfx DNA Polymerase (Life Technologies) will be used for DNA amplification by PCR following manufacturer's instructions. qPCR will be carried out using the Twist Biosciences SYBR Green Real-Time PCR Master Mix.

Plant and microbial growth conditions

Nicotiana benthamiana plants will be grown in a glasshouse at 25°C with a photoperiod of 12-h day/12-h night and 60% relative humidity.

Pseudomonas syringae pv. tomato (Pst) DC3000 and Bacillus subtilis will be grown on LB solid medium at 28°C, while Bacillus subtilis (Bst) will be grown at 32°C on the same medium.

Bacterial transformation

Bacteria will be transformed with plasmids harbouring mCherry and mVenus respectively and antibiotic resistance genes. Electrocompetent cells will be prepared for both. Bst will be grown in flasks at 37℃ at 250 rpm until an OD600 of 0.7 will be reached with three samples 20% glycine solution with concentrations of 1, 1.25 and 1.5 respectively being added and aliquots and frozen. The aliquots will be then electroporated with 600ng DNA. Pst will be prepared at 28°C and grown in a flask until OD600= 0.5, centrifuged at 5,500 rpm and in decreasing amounts of 0.5 M sucrose solution for competent cell preparation. They will be then electroporated with 150ng DNA at 5 kV/cm Fluorescent colonies will be selected using an LED in the appropriate wavelength to excite mCherry and mVenus and will be grown overnight in LB agar medium at the aforementioned temperatures and containing the appropriate antibiotics: 100mg/ml diluted to 1/1000 spectinomycin and 50 mg/ml diluted to 1/1000 kanamycin will be used to select for Bst and Pst respectively.

Agroinoculation of N. benthamiana leaves

The strain GV3101:pMP90 of A. tumefaciens, which harbors the helper plasmid pCLEAN-S4844, will be transformed chemically with pRepress and pActivate, bearing modCas9 and mod-Cas9 and SoxS respectively with the necessary resistance genes included. Chemically competent cells will be used. Aliquots will be thawed and 500ng of DNA will be used, incubated at 37°C in a water bath, and then added to 950 µl of recovery media and incubated at 200 rpm in a shaking incubator at 28°C. The transformed colonies will be selected in plates with a lysogenic broth (LB) medium containing 50 µg/ml rifampicin. The presence of the plasmid will be verified by colony PCR after selection. Liquid cultures will be grown for 24 h at 28 °C in the same medium. Bacteria will be recovered via centrifugation and brought to an optical density at 600 nm of 0.5 in agroinoculation solution (10 mM MES-NaOH, pH 5.6, 10 mM MgCl2 and 150 µM acetosyringone). The cultures will be incubated for 2 h at 28 ℃ to induce the virulence genes, and used to infiltrate the leaves of 5-week old N. benthamiana plants. Each A. tumefaciens culture will be brought to an optical density of 0.1. A. tumefaciens cultures will be infiltrated at the abaxial side of the leaves using a needleless 1-mL syringe. The infiltrated plants will be cultured in a growth chamber at 25ºC with a 12-h day-night photoperiod for 24-48 hours.

Hairy Root Transformation

A. rhizogenes ATCC 15834 will be electroporated with prepress and pActivate using electroporation. An A. rhizogenes culture harboring the binary vector will be grown overnight at 28 °C on a rotary shaker at 170 rpm to mid-log phase (OD600 1) in 10 mL liquid LB medium supplemented with 50 mg/L rifampicin. The bacterial cells will be collected by centrifugation at 3,000g for 10 min and the bacterial pellets will be re-suspended to an OD600 0.5-1 in MS (Murashige and Skoog, 1962) liquid medium without sugar before plant inoculation. Mature leaves will be grown aseptically on solid MS medium, containing 3 g/L sucrose (pH 5.8) and then will be cut into 1 cm2 length and transferred onto an suspension of A. rhizogenes and incubated for 5 min with gentle shaking. The bacteria will be poured off and the explants will be dried on sterile filter paper and placed on MS solid medium (containing 3 g/L sucrose, pH 5.8) in so that the surface will be facing the medium for 2 days in darkness. After two days of inoculation with bacteria, the explants will be placed onto new MS plates containing antibiotics until hairy root tips appeared. The explants will be subcultured on new MS antibiotic plates continuously. The hairy root tips (4-5 cm) will be cut off then transferred separately to a new MS plate. The tobacco HR will be kept in these conditions at 25 °C in the dark for several rounds of subculturing until the elimination of agrobacteria and confirmation by PCR analysis

Inoculation with Pst and Bst

Plants will be inoculated with Pst 48 hours after agroinoculation by inoculating one colony into 10 ml LB medium, centrifuging the overnight culture, resuspension of the pellet resuspension of the pellet in MgCl2 10mM to an OD600 of 0.2 and dipping leaves in the suspension with 0.02% Silwet L-77. Plants will be inoculated with Bst similarly but added to the MS medium in one group and leaves in a second group to mitigate distortion of results due to hairy roots and without surfactant, diluted to 10^8 CFU in 0.85% NaCl saline solution. A control group will be set up inoculated with 0.85% NaCl solution or 10 mM MgCl2.

Preparation of leaf samples and confocal laser-scanning microscopy

Fresh leaf disks of 2.2 cm in diameter will be harvested 3 days post infiltration (dpi). These disks will be imaged with a Leica SP8 confocal laser-scanning microscope (Leica Microsystems, Australia) equipped with a 40× (NA = 1.1) water immersion objective. Images will be acquired with the

Leica LASX software. mTurquoise2, mVenus and mCherry will be excited at 458, 515 and 587 nm respectively and filters for 474, 527, and 610 nm respectively will be used.

Isolation of mod-dCas9 in AWF (Apoplastic Wash Fluid) Fractions From Infiltrated N. benthamiana Leaves

The mod-dCas9 will be isolated from AWF fractions as described previously (Alkanaimsh et al., 2016). Agro-Infiltrated fresh leaves will be harvested at 3 dpi and submerged in a harvest buffer, TBS-2 (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% Silwet L-77) in a glass dish, and subsequently placed in a vacuum chamber for a 1 min vacuum infiltration. The infiltrated leaves will be

transferred into 50 ml conical-bottom centrifuge tubes, and centrifuged for 10 min at 4°C at 1000g. The AWF fractions was recovered as the supernatants. One N. benthamiana leaf yields 300–400 µl of AWF, which was subsequently 4-fold concentrated through acetone precipitation for immunoblotting.

Immunoblotting Assay

Total proteins were extracted from agro-infiltrated leaves at 5 DPI with 1:2.5 (w/v) protein extraction buffer (50 mM Tris–HCl, pH 8.0, 10 mM KCl, 10 mM MgCl2, 1 mM EDTA, 20% glycerol, 2% SDS and 10% β-Mercaptoethanol). Extracted total proteins were separated by electrophoresis through a 12% polyacrylamide gel containing 0.1% SDS. The IB analysis used α-GFP antibodies to detect the mTurquoise2 tag fused to mod-dCas9 and α-RFP antibodies were used for the determination of expression levels of mCherry and mVenus in the bacteria before N. benthamiana inoculation and following modulation by mod-dCas9 expression. Overlap is not expected due to the differing protein sizes of mCherry, mVenus and mod-Cas9.

Biosafety

Our research work was in accordance with the guidelines presented by GOGEC 2025. It was made sure that all the work that was being done was within the guidelines of GOGEC. All experiments with genetically modified organisms (GMOs), Nicotiana benthamiana, and bacterial strains were conducted in compliance with biosafety guidelines. Agroinfiltration, fluorescent marker use, and handling of pathogens like Pseudomonas syringae and

Bacillus subtilis were performed in containment facilities with appropriate PPE. Hazardous chemicals, including β-mercaptoethanol and SDS, were handled following safety protocols, and waste was sterilized to prevent environmental exposure .Furthermore, it was made sure that fluorescent marker-containing bacteria and plant samples were managed in compliance with lab protocols

Human Centered Design

Agriculture plays a pivotal role in the growth of a society whether in terms of food supply, providing of raw or the use of goods to establish a country's economy; it is extremely important that we ensure to improve agricultural practices. These plants offer an incredible way to improve the agricultural practices, providing farmers with good quality seed that not only could boost their crop yield but would also help provide financial security to farmers. However, it is essential that we remain mindful about the effect of advancement on local communities, other crops and the environment. The government can implement strict policies on biotechnology companies to ensure that no ethical or moral implications arise with respect to the use of GM plants and its seeds. To our knowledge, the major concerns of farmers revolve around being sued for seed piracy, either due to transgene escape, the prevention of which further burdens them economically, or due to harvesting natural seeds, which forces them to purchase new seeds each harvest season. It is paramount that regulations and patents are put in place to protect our project from such misuse. Moreover, educational campaigns can be carried around where the farmers could be informed about their rights and could also learn more about the growth of GM crops to guarantee agricultural growth.

Expected results

According to various studies it has been suggested that change in fluorescence intensity is highly dependent on the activation or repression of the target fluorescent protein. The activation using dCas9-based systems should increase the fluorescence intensity while repression of the protein should result in a decrease in the fluorescence intensity. These expected results are based on previous investigations that have been carried out; It has been suggested that the use of dCas9- SoxS increases the fluorescence activity up to 20 folds mainly relying on the transcriptional repressor and the target gene. These findings are quite consistent as compared to various other investigations that have been

carried out and ensure that the increase in fluorescent light can be a direct indicator of gene expression. On the other hand, the dCas9-based repressors show a decrease in fluorescence of about 3-5 folds. Hence, the gene expression in GM plants is expected to show a direct correlation with the activity of fluorescent light.

CONCLUSION

We have developed a proposal to engineer a host-to-bacterium protein secretion system in Nicotiana benthamiana. We hope that we will be able to develop our proof-of-concept fully and that this proposal and can be translated to real-world GM seeds that can pave the way for more resilient, sustainable agriculture.

References

Barrick Lab. Working with Pseudomonas Syringae. Barrick Labs, 6 July 2016, https://barricklab.org/twiki/bin/view/Lab/ProtocolsWorkingWithPseudomonasSyringae .

Bassett, Carole L., and Wojciech J. Janisiewicz. "Electroporation and Stable Maintenance of Plasmid DNAs in a Biocontrol Strain of Pseudomonas Syringae." Biotechnology Letters, vol. 25, no. 3, Feb. 2003, pp. 199–203. Springer Link, https://doi.org/10.1023/A:1022394716305.

Chahardoli, Mahmood, et al. "Recombinant Production of Bovine Lactoferrin-Derived Antimicrobial Peptide in Tobacco Hairy Roots Expression System." Plant Physiology and Biochemistry, vol. 123, Feb. 2018, pp. 414–21. DOI.org (Crossref), https://doi.org/10.1016/j.plaphy.2017.12.037.

Chen, Kai, et al. "Engineering Self-Deliverable Ribonucleoproteins for Genome Editing in the Brain." Nature Communications, vol. 15, no. 1, Feb. 2024, p. 1727. www.nature.com, https://doi.org/10.1038/s41467-024-45998-2.

Compant, Stéphane, et al. "Harnessing the Plant Microbiome for Sustainable Crop Production." Nature Reviews Microbiology, vol. 23, no. 1, Jan. 2025, pp. 9–23. DOI.org (Crossref), https://doi.org/10.1038/s41579-024-01079-1.

Gadhave, Kiran R., et al. "Soil Inoculation with Bacillus Spp. Modifies Root Endophytic Bacterial Diversity, Evenness, and Community Composition in a Context-Specific Manner." Microbial Ecology, vol. 76, no. 3, 2018, pp. 741–50. PubMed Central, https://doi.org/10.1007/s00248-018-1160-x.

GoldBio. Chemicall Competent Cell Preparation. GoldBio Technologies, 7 May 2021, *https://goldbio.com/documents/4373/GV3101%20Agrobacterium%20Chemically%20Competent%20Cells%20Protocol.pdf*.

Hanif, Muhammad Sajid, et al. *"Plant Microbiome Technology for Sustainable Agriculture."* Frontiers in Microbiology, vol. 15, Nov. 2024. Frontiers, https://doi.org/10.3389/fmicb.2024.1500260.

Jacob, Cristián, et al. "Surface Inoculation and Quantification of Pseudomonas Syringae Population in the Arabidopsis Leaf Apoplast." Bio-Protocol, vol. 7, no. 5, Mar. 2017, p. e2167. PubMed Central, https://doi.org/10.21769/BioProtoc.2167.

Jiang, Min-Chao, et al. "Fusion of a Novel Native Signal Peptide Enhanced the Secretion and Solubility of Bioactive Human Interferon Gamma Glycoproteins in Nicotiana Benthamiana Using the Bamboo Mosaic Virus-Based Expression System." Frontiers in Plant Science, vol. 11, Nov. 2020. Frontiers, https://doi.org/10.3389/fpls.2020.594758.

Nagano, Minoru, et al. "Generation of Arabidopsis Lines with a Red Fluorescent Marker for Endoplasmic Reticulum Using a Tail-Anchored Protein Cytochrome b5 -B." Plant

Signaling & Behavior, vol. 15, no. 9, Sept. 2020, p. 1790196. DOI.org (Crossref), https://doi.org/10.1080/15592324.2020.1790196.

O'Leary, Brendan M., et al. "The Infiltration-Centrifugation Technique for Extraction of Apoplastic Fluid from Plant Leaves Using Phaseolus Vulgaris as an Example." Journal of Visualized Experiments (JoVE), no. 94, Dec. 2014, p. e52113. app.jove.com, https://doi.org/10.3791/52113.

Stoddard, Angela, and Vivien Rolland. "I See the Light! Fluorescent Proteins Suitable for Cell Wall/Apoplast Targeting in Nicotiana Benthamiana Leaves." Plant Direct, vol. 3, no. 1, Jan. 2019, p. e00112. DOI.org (Crossref), https://doi.org/10.1002/pld3.112.

Talens-Perales, David, et al. "Expression of an Extremophilic Xylanase in Nicotiana Benthamiana and Its Use for the Production of Prebiotic Xylooligosaccharides." Scientific Reports, vol. 12, no. 1, Sept. 2022, p. 15743. www.nature.com, https://doi.org/10.1038/s41598-022-19774-5.

UBonn, and Freiburg iGEM 2016. How to Bacillus Subtilis. UBonn iGEM 2016, 2016, https://static.igem.org/mediawiki/2016/7/74/T--UBonn_HBRS--How-To-Bacillus-Subtilis.pdf.

Xin, Xiu-Fang, et al. "Pseudomonas Syringae: What It Takes to Be a Pathogen." Nature Reviews Microbiology, vol. 16, no. 5, May 2018, pp. 316–28. www.nature.com, https://doi.org/10.1038/nrmicro.2018.17.

Yadav, Ajar Nath. "Plant Microbiomes for Sustainable Agriculture: Current Research and Future Challenges." Plant Microbiomes for Sustainable Agriculture, edited by Ajar Nath Yadav et al., Springer International Publishing, 2020, pp. 475–82. Springer Link, https://doi.org/10.1007/978-3-030-38453-1_16.

Zhang, Yifan, et al. "A Nicotiana Benthamiana Receptor-like Kinase Regulates Phytophthora Resistance by Coupling with BAK1 to Enhance Elicitin-triggered Immunity." Journal of Integrative Plant Biology, vol. 65, no. 6, June 2023, pp. 1553–65. DOI.org (Crossref), https://doi.org/10.1111/jipb.13458.

Chari, Raj, et al. "sgRNA Scorer 2.0 – a Species Independent Model to Predict CRISPR/Cas9 Activity." ACS Synthetic Biology, vol. 6, no. 5, May 2017, pp. 902–04. PubMed Central, https://doi.org/10.1021/acssynbio.6b00343.

Cell Biology 04: The Secretory Pathway. https://www.cureffi.org/2013/02/24/cell-biology-04-the-secretory-pathway/#:~:text=The%20secretory%20pathway%20refers%20to%20the%20endoplasmic%20reticulum%2C,the%20cell%20secretes%20proteins%20into%20the%20extracellular%20environment. Accessed 25 Jan. 2025.

Agrawal, Shilpi, et al. "Overexpression and Cell-Penetrating Peptide-Mediated Delivery of Cas9 and Its Variant(s) for Targeted Genome Editing." Biophysical Journal, vol. 122, no. 3, Feb. 2023, pp. 434a–5a. DOI.org (Crossref), https://doi.org/10.1016/j.bpj.2022.11.2349.

Sadiq, Idris Zubairu, et al. "Biotherapeutic Effect of Cell-Penetrating Peptides against Microbial Agents: A Review." Tissue Barriers, vol. 10, no. 3, July 2022, p. 1995285. DOI.org (Crossref), https://doi.org/10.1080/21688370.2021.1995285.

Borrelli, Antonella, et al. "Cell Penetrating Peptides as Molecular Carriers for Anti-Cancer Agents." Molecules : A Journal of Synthetic Chemistry and Natural Product Chemistry, vol. 23, no. 2, Jan. 2018, p. 295. PubMed Central, https://doi.org/10.3390/molecules23020295.

Chavez, A., Tuttle, M., & Choi, Y. (2015). "Comparison of CRISPR/Cas9, TALENs, and

ZFNs for gene editing." Nature Biotechnology, 33(2), 150-156. https://doi.org/10.1038/nbt.3117

Gilbert, L. A., Horlbeck, M. A., & Adamson, B. (2013). "Genome-scale CRISPR-mediated control of gene repression and activation." Cell, 159(3), 647-661. https://doi.org/10.1016/j.cell.2014.09.029

Ouyang, Jun, et al. “Recent Advances of Studies on Cell-Penetrating Peptides Based on Molecular Dynamics Simulations.” Cells, vol. 11, no. 24, Dec. 2022, p. 4016. PubMed Central, https://doi.org/10.3390/cells11244016.