

In-Depth Exploration of CRISPR Technology and Recent Discoveries in Gene Editing Tools: Silencing the lacZ Gene in E. Coli using Bacteria's Natural Prokaryotic Defense Mechanisms

ERA JOY SMITH, MEGHAA SHANMUGAM, ALEX ROCKWELL, YASMITHA ANDRA, MARVELLA ABDELMALEK, DR. SAIRAM RUDRABHATLA, DR. SHOBHA RUDRABHATLA

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ONE BRIGHT RAY COMMUNITY HIGH SCHOOL, PHILADELPHIA, PA 19121, PENN STATE HARRISBURG, BIOFUELS LABORATORY, MIDDLETOWN, PA 17057

ABSTRACT

A shrinking global breadbasket threatens future generations with the hunger pains of potential starvation and malnutrition. Genetic engineers and molecular biologists are using a revolutionary genetic technology to manipulate and edit the genomes of various species of the plantae and animalia kingdoms fostering gene expression of more favorable and sustainable traits. Clustered regularly interspaced short palindromic repeats (CRISPR) DNA sequences were discovered in archaea and bacteria in 2002 during exploratory bioinformatic analyses. Genes discovered in close proximity to these CRISPR sequences were named as CRISPR associated genes ("Cas" genes). The CRISPR Cas system is a genome editing technology that allows for the insertion of beneficial genes of one species to another while "knocking out" or minimizing less desirable traits. The research conducted this summer sought to identify the processes and methodology involved in gene editing of a common gene under study as well as exploring new developments in gene editing technologies.

INTRODUCTION

The two-part technological system, known as CRISPR Cas9, uses a Cas-associated protein (Cas9) and a single guide ribonucleic acid (sgRNA), also known as a CRISPR RNA (crRNA) molecule, to make an intentional molecular cut of the DNA strand thereby activating the cell's natural repair system, either by homology directed repair (HDR) or non-homologous end joining (NHEJ). Once the CRISPR Cas complex (Cas9 enzyme and sgRNA) locates and cleaves the target sequence, the desirable genetic code is supplanted in place of the "knocked-out" gene. Genomic editing technologies like the CRISPR/Cas system rely on exploiting the natural defense mechanisms of bacterial and archaeal cell physiology. Subsequently, the rise of new bioinformatics and AI-assisted omics applications have uncovered areas for research on the use of newly discovered Cas proteins and other programmable RNA insertion sequences (IS) like seekRNA that attempts to maximize guide RNA programmability and minimize off-target results. Gene modification technologies may lead to improved human/plant health and genetic wellness.

METHODOLOGY

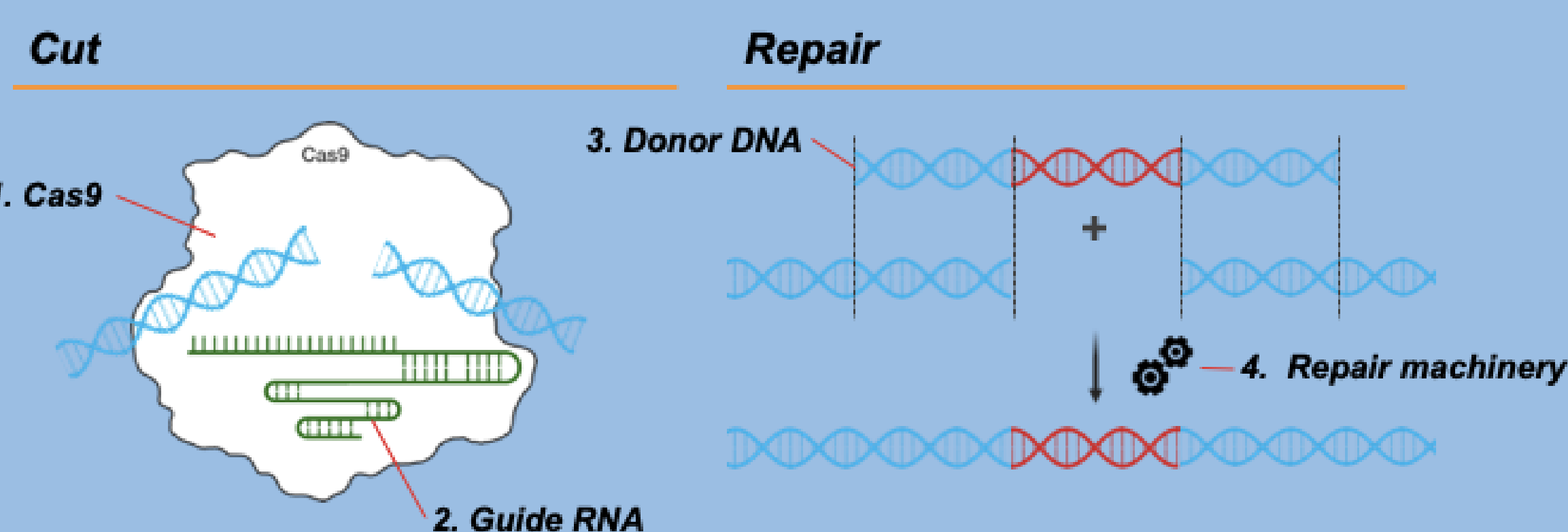
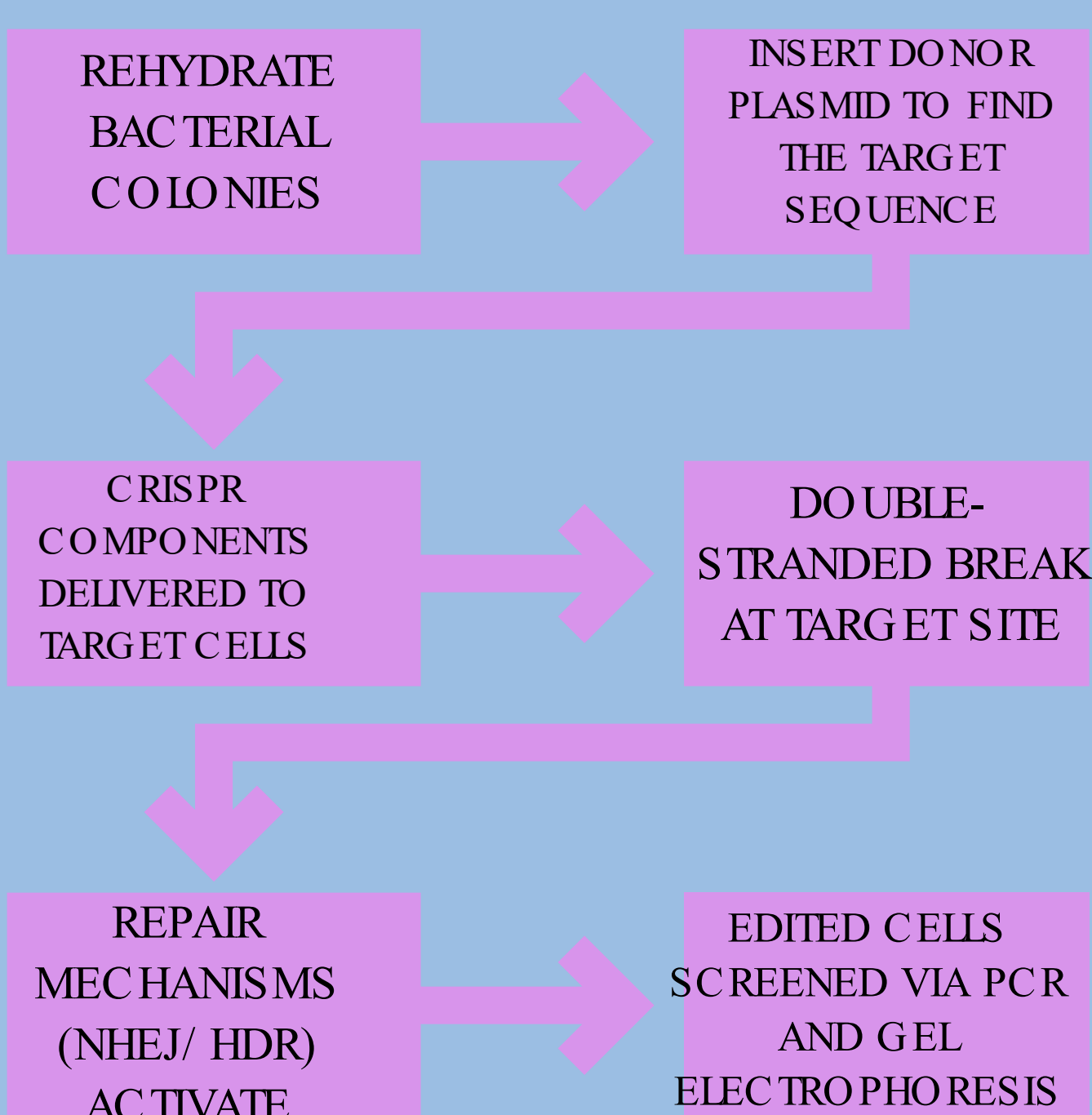


Figure 1: Diagram and CRISPR system components courtesy of Bio-Rad Laboratories, Inc.

RESULTS

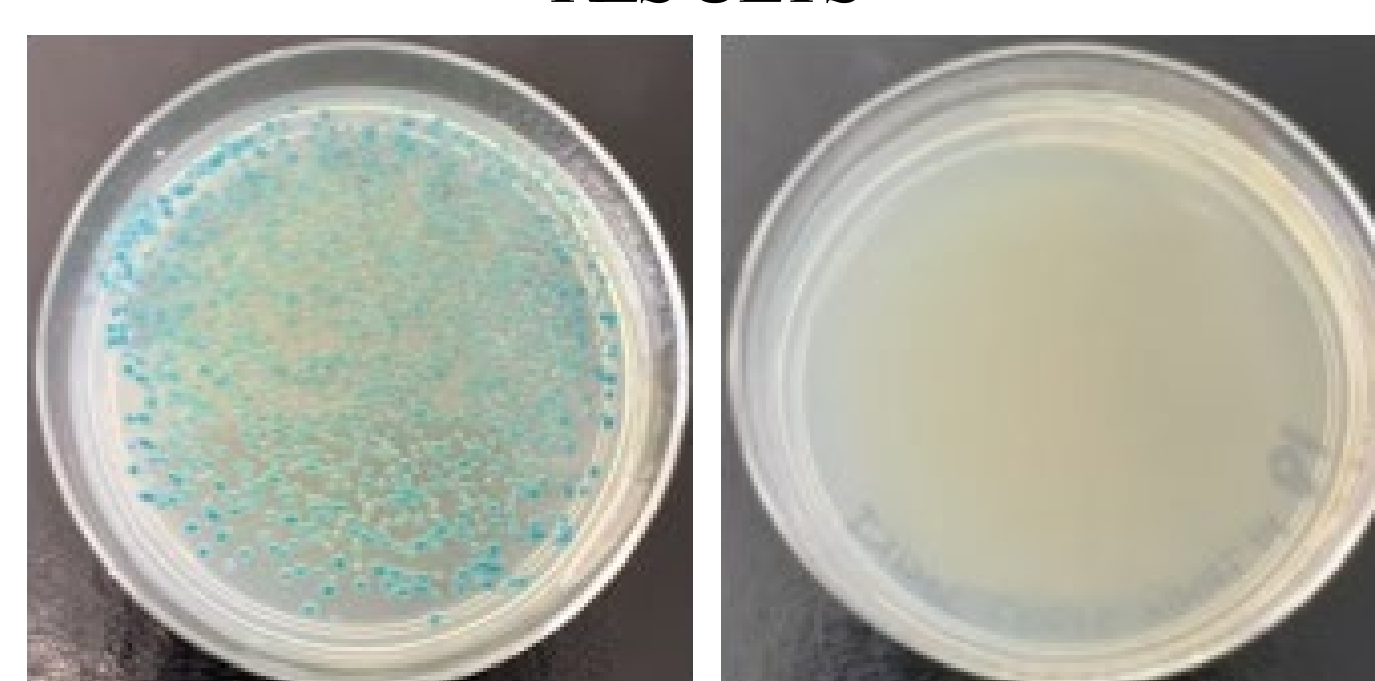


Figure 2: Plate A, grown without arabinose and containing the donor plasmid (pLZDonor), shows blue colonies due to the hydrolysis of X-gal by β -gal, indicating no double-strand breaks or repair necessity. Plate B lacked arabinose and contained the pLZDonorGuide plasmid with a single-guide RNA for Cas9 to make double-stranded DNA cuts. Without arabinose, cells could not use their repair mechanism, resulting in no colonies.

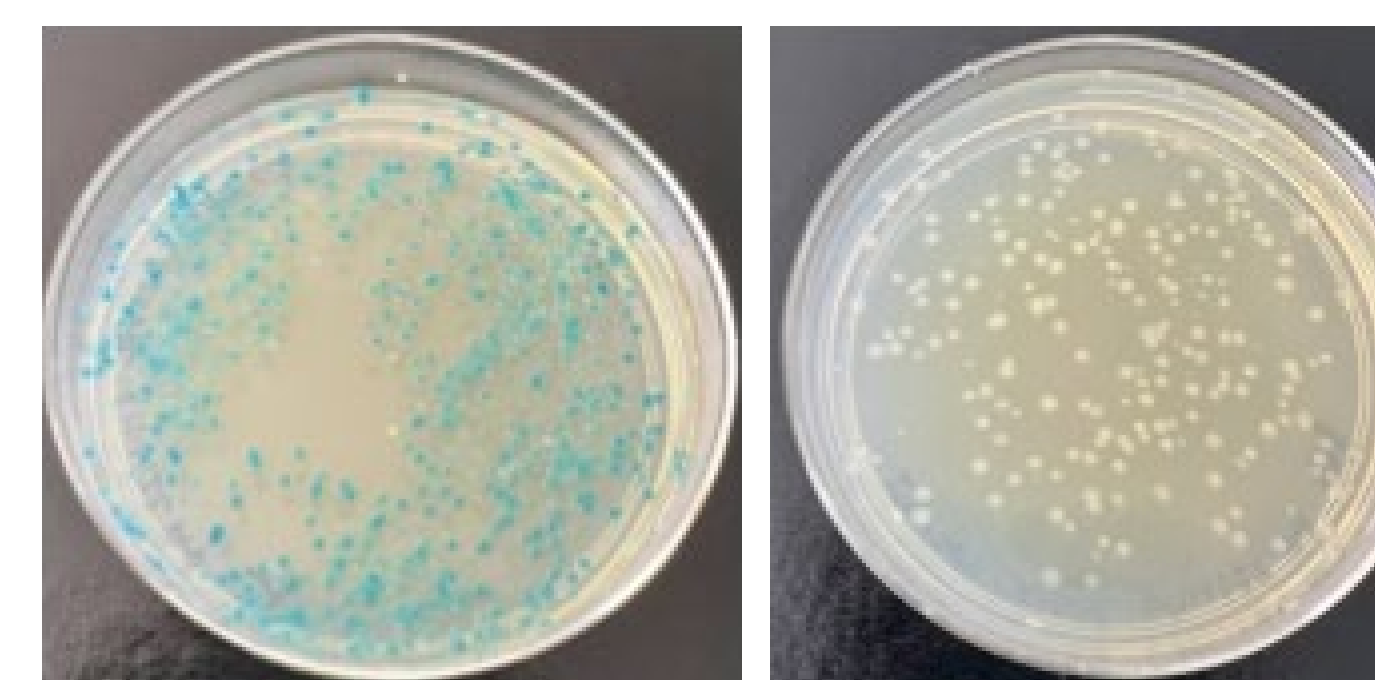


Figure 3: Plate C, grown with arabinose and containing the pLZDonor plasmid, showed blue colonies due to β -gal production from X-gal hydrolysis, indicating no double-stranded breaks and no need for repair. Unlike Plate A, Plate C had an arabinose-inducible promoter for potential repair of double-stranded breaks. Plate D, containing the pLZDonorGuide plasmid and grown with arabinose, showed white colonies due to successful knockout of the lacZ gene by the single-guide RNA and repair of DNA breaks facilitated by arabinose. The absence of blue pigment confirms the knockout.

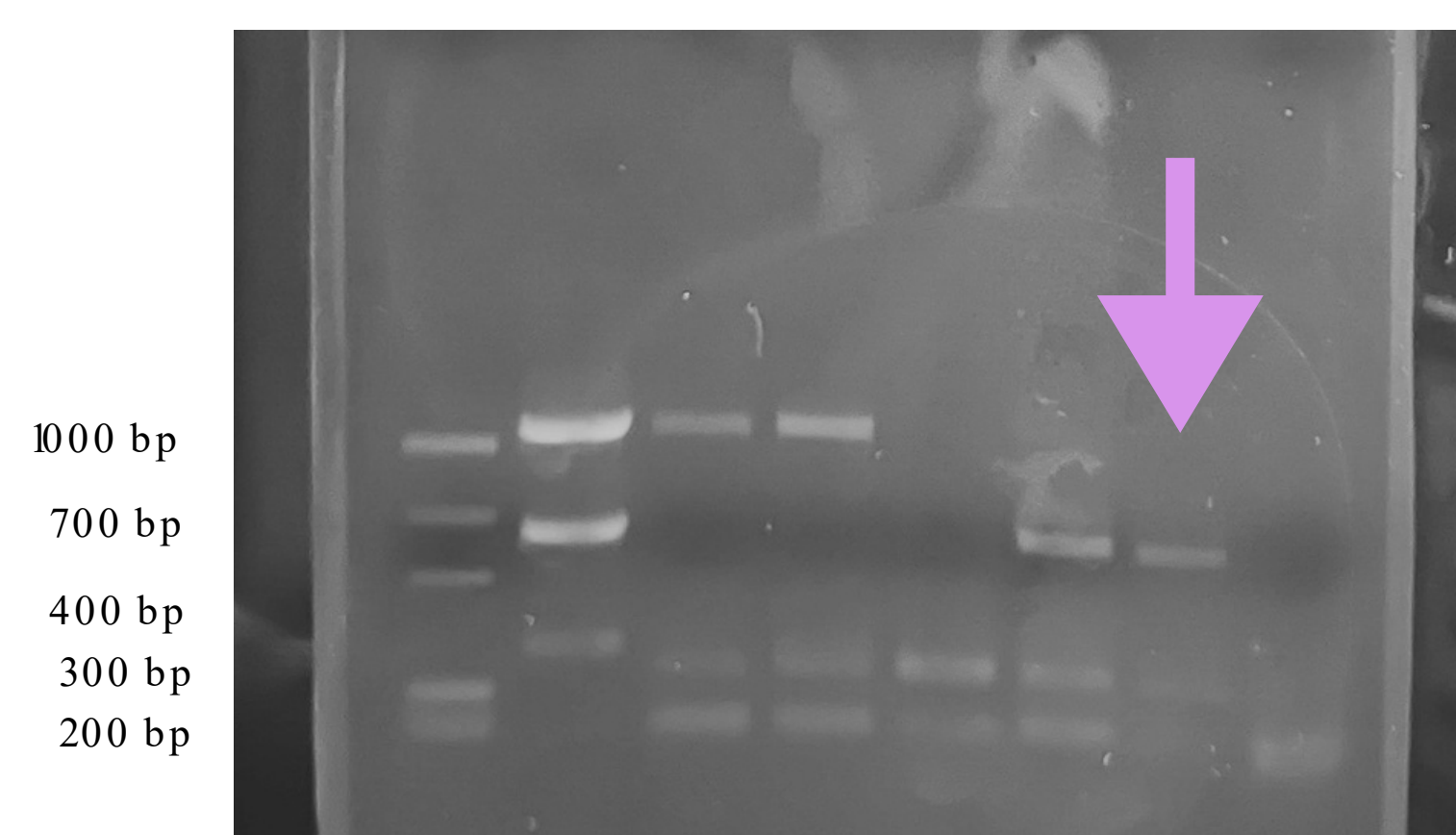


Figure 4: Gel electrophoresis results under UV light with knocked lacZ gene highlighted.

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Cas systems	Class 2, type II	Class 2, type V-A	Class 2, type V-B	Class 2, type V-J	Class 1, type I
Features					
Cas protein requirement	Cas9	Cas12a (Cpf1)	Cas12b (C2c1)	Cas12j (CasΦ)	Cascade complex + Cas3
Cas protein size	1000-1400 aa	1100-1300 aa	1100-1500 aa	700-800 aa	-
RNA requirement	crRNA + tracrRNA	crRNA	crRNA + tracrRNA	crRNA	crRNA
PAM	3' PAM, G-C rich	5' PAM, A-T rich	5' PAM, A-T rich	5' PAM, A-T rich	5' PAM, A-T rich
Nuclease domain	HNH + RuvC	RuvC	RuvC	RuvC	HD
Cleavage pattern	Blunt end	Staggered end	Staggered end	Staggered end	Various
Predominant editing events	1 bp or small InDels	Multi-nucleotide deletion	4-14-bp deletion	8-10-bp deletion	100-bp-100-kb deletion
Multiplexing capability	High	High	Unknown	Unknown	Unknown
Current applications	Knock-out, knock-in, base editing, prime editing, epigenetic editing, gene regulation...	Knock-out, knock-in, gene regulation...	Knock-out, knock-in, gene regulation...	Knock-out, promoter editing, gene regulation...	Knock-out, gene regulation...
Applicable in promoter editing	Currently the main tool, adapt almost all tasks without efficiency consideration	An equally effective tool with clear advantages, especially in non-coding regions like promoters	Could be promising promoter editing tools and worth further exploration	Could be promising promoter editing tools and worth further exploration	A multi-component editor with limited application, but has relative advantages in large-scale editing

Figure 5: Chart of alternate CRISPR-associated (Cas) protein systems, highlighting their most prominent features.

Bioinformatics Databases Use for Gene Editing Efficiency and Accuracy

Genomic Databases	CRISPR Databases	Variants/Mutations Databases	Functional Annotation Databases	Protein Databases	Off-Target Prediction Tools
<ul style="list-style-type: none"> Ensembl: Provides comprehensive genetic information on numerous species. Includes regulatory elements, variants, and gene structures. UCSC Genome Browser: Visual interactive genomic data platform inclusive of gene annotations, variants, and epigenetic modifications across multiple species. Genome: A curated, integrated, open source database where comparative functional genomics on crops and model plant species can be found. 	<ul style="list-style-type: none"> CRISPRpedia: The functionalities and applications in gene editing of CRISPR-associated genes and CRISPR-Cas systems can be found here. The Bio Institute's CRISPR Database: CRISPR-Cas9 system database that informs about target sites, inclusive of predicted off-target effects and a tool for designing guide-DNA molecules. Gene: Offers gene editing experimental design tools along with a CRISPR-Cas9 target sites and off-target predictions tool. 	<ul style="list-style-type: none"> dbSNP: Database of single nucleotide polymorphisms (SNPs) and small genetic variations within species populations. Clincat: Database providing information on genetic variants and human health relationships, inclusive of those of clinical significance and pathogenicity. COSSMC: Catalogue Of Somatic Mutations In Cancer useful in comprehending genetic changes associated with tumors. 	<ul style="list-style-type: none"> Gene Ontology (GO): Offers a framework representation of genetic functions and relationships, inclusive of their biological processes, molecular functions, and cellular components. KEGG: The Kyoto Encyclopedia provides data on metabolic and biological pathways to understand gene functions and interactions. Reactions: Free open-space database and biological pathway and reactions, offering comprehensive information on molecular interactions and pathway analysis. 	<ul style="list-style-type: none"> UniProt: In-depth database on protein sequences and functions, including annotations on post-translational modifications and interactions. PDB: The Protein Data Bank contains 3D structures of proteins and macromolecules aids in understanding of structural implications of genomic editing. 	<ul style="list-style-type: none"> CRISPR: Tool that can aid in designing CRISPR guide RNAs and used to predict potential off-target effects based on genomic sequences. Off-Target: An alternative to the term "off target" this tool is used to predict potential off-target sites for gene editing technology and helping to evaluate the specificity of guide RNA.

Figure 6: A Comprehensive Overview of Leading Bioinformatics Databases

CONCLUSION

1. CRISPR Effectiveness: CRISPR/Cas9 with pLZDonorGuide successfully knocked out the lacZ gene, as shown by white colonies on Plate D.
2. Repair Activation: HDR repair was effective with arabinose (Plate D) but not without it (Plate B), demonstrating that repair mechanisms are crucial for CRISPR/Cas9 gene editing.
3. Single-Guide RNA: The single-guide RNA effectively directed Cas9 to lacZ, causing a DSB and gene knockout. When HDR repair mechanisms were active, the cell survived.
4. Arabinose Role: Arabinose is essential for activating repair pathways and achieving successful gene editing with CRISPR/Cas9, without it the cell would undergo apoptosis due to the DSB.

REFERENCES

1. Siddique, R. (2024) "A programmable seekRNA guides target selection by IS1111 and IS110 type insertion sequences" Nature Communications 15:5235 1-15
2. Tang, X. and Zhang, Y. (2023). Beyond knockouts: fine-tuning regulation of gene expression in plants with CRISPR-Cas-based promoter editing. New Phytol. 239: 868-874. <https://doi.org/10.1111/nph.19020>
3. Ahmad, A., Jamil, A., Munawar, N. GMOs or non-GMOs? The CRISPR Conundrum. Front Plant Sci. 2023 Oct 9;14:1232938. doi: 10.3389/fpls.2023.1232938. PMID: 37877083; PMCID: PMC10591184.