



**NATIONAL RESEARCH PROGRAM FOR UNIVERSITIES (NRPU)
PROPOSAL COVER SHEET**

Proposal Reference No. (Not for completion by applicant)	
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Title of Project:	TARGETED mRNA DEMETHYLATION IN MAIZE USING AN ENGINEERED dCAS13a-ALKBH5 FUSION PROTEIN	
Duration of Project:	3 years	
Total Budget Requested	PKR million	
Theme of Proposed Research	Basic <input checked="" type="checkbox"/>	Applied <input type="checkbox"/>
Discipline of Proposed Research	Bioengineering and Technology	
Major Field	Biotechnology and Bioengineering	
Minor Field	Plant Biology	

To which priority area of national relevance does the proposal respond?	Enhancing crop resilience through precise RNA modification (demethylation) in maize.
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II. EXECUTIVE SUMMARY

The role of epigenetic modifications in genome evolution and innovation has been predominantly attributed to DNA modifications, leaving the influence of posttranscriptional RNA modifications relatively understudied. In an effort to delve into the evolutionary significance of RNA modifications, extensive transcriptome-wide profiles of N6-methyladenosine (m6A), the primary internal modification found in mRNA, were generated. N6-methyladenosine (m6A) is an important epigenetic modification that is involved in RNA stability and to enhance translation efficiency. For modification of mRNA m6A methyltransferases or demethylases are used that makes it difficult to study the effect of specific RNA methylation. N6-Methyladenosine (m6A) stands as a pivotal and dynamic modification found within mRNA molecules, persistently observed in diverse transcripts. The presence of m6A modification has been discerned in numerous RNA transcripts in maize, exhibiting distinct distribution patterns. In the realm of maize plants, mRNA undergoes reversible modifications facilitated by a methyltransferase complex (e.g., METTL3/METTL4/WTAP, termed as 'writers') and demethylases ('erasers' such as FTO and/or ALKBH5) that intricately regulate the m6A modification. The project aims to investigate and manipulate mRNA modification in maize using a tailored CRISPR–Cas13b-based tool. It delves into the intricate world of RNA methylation, particularly focusing on m6A modification, a pivotal regulatory mechanism influencing gene expression. By creating a fusion protein (dm6ACRISPR) combining an inactive Cas13b enzyme with the m6A demethylase ALKBH5, the study endeavors to precisely target and alter RNA methylation in maize cells. In this study, we reported the development of a construct using PspCas13b (from *Prevotella* sp.) and human m6A demethylase ALKBH5 catalytic domain. This construct specifically demethylates m6A of targeted mRNAs to enhance mRNAs stability. Due to advances in transcriptome-wide m6A sequencing and mapping technology, m6A modification has been demonstrated to have pivotal roles in biological and developmental processes of plants, such as embryo development, shoot apical meristem development, fruit ripening, enhanced resistance, and response to stresses. The study focuses on targeted mRNA demethylation in maize using a newly engineered tool, dm6ACRISPR, comprising a fusion of the dPspCas13b enzyme and the m6A demethylase ALKBH5. Maize serves as an ideal model to investigate gene expression regulation via epigenetics, specifically RNA modifications. The researchers designed the dm6ACRISPR system and validated its efficacy in demethylating specific mRNA targets in maize cells. They detailed the construction of the tool, guide RNA design, protoplast isolation, transfection procedures, and subsequent analyses, showcasing its ability to influence mRNA stability, selectively demethylate identified m6A sites, and modulate gene expression. This precise RNA modification method highlights m6A's regulatory role in maize gene expression, promising advancements in plant biology and potential agricultural biotechnology applications for crop improvement. Overall, this project pioneers a precise and targeted approach to modify RNA methylation, offering insights into the regulatory mechanisms of gene expression in maize. Its implications extend from basic biology of plants to possible uses in agricultural biotechnology for managing stress responses and improving crop yields.

III. PRIORITY AREA OF NATIONAL RELEVANCE, AND IMPACT OF PROPOSED NRPU PROJECT ON THE PRIORITY AREA

This paper describes a novel approach to precisely target and change RNA methylation in maize plants using the CRISPR/Cas13 system. The science of epigenetics will benefit greatly from this discovery, especially in understanding how RNA changes in plants control gene expression.

This work is significant because it provides a highly accurate method for analyzing and adjusting RNA methylation at particular loci in the maize genome. Unlike existing methods that induce broad changes in RNA methylation, this targeted approach allows for precise modifications, avoiding widespread epigenetic alterations. By engineering a fusion protein (dm6ACRISPR) composed of a catalytically inactive Cas13b enzyme fused with the m6A demethylase ALKBH5, researchers can demethylate specific mRNA sequences in maize cells.

The significance of this research extends to various aspects:

Understanding Epitranscriptomic: First detected in the RNA of calf liver, chemical modification of RNA was later revealed to be present in all living forms. Over 160 different types of RNA modifications have been identified; the majority of these types are found in ribosomal RNA (rRNA) and transfer RNA (tRNA). The first known mRNA alteration was called m6A, and it was discovered in mammals before spreading to other eukaryotic species like plants. This method allows a deeper understanding of how RNA modifications, specifically m6A methylation, influence gene expression, mRNA stability, translation efficiency, and protein synthesis in maize plants.

Targeted RNA Modification: dCas9 has been employed in human studies to modify epigenetic properties such as DNA methylation and histone modifications, recent research has highlighted the potential of dCas13b-m6A reader fusion proteins to target specific transcripts in maize, regulating mRNA translation and degradation. In this study, we aim to construct and characterize a CRISPR–Cas13b-based tool tailored to target the demethylation of specific mRNA in maize. By fusing the catalytically inactive Type VI-B Cas13 enzyme from *Prevotella* sp. P5–125 (dPspCas13b) with the m6A demethylase ALKBH5 by using a linker, we demonstrate the effectiveness of our engineered construct, named dm⁶ACRISPR, in successfully demethylating targeted mRNA in maize cells. Furthermore, we showcase the utility of the dm⁶ACRISPR system in investigating the regulatory influence of m6A methylation on specific endogenous mRNA in maize.

ALKBH5 is a gene that encodes for a protein involved in the modification of RNA molecules. This protein is part of a family of enzymes known as AlkB homologs, which play a role in removing specific chemical modifications (such as m6A) from RNA molecules. ALKBH5, specifically, is associated with the demethylation of N6-methyladenosine (m6A) modifications in RNA, impacting various processes like gene expression regulation and mRNA stability in organisms, including plants.

Specifically, the development and application of a novel system, **dm6ACRISPR**, to enable targeted demethylation of specific mRNA in maize plants. In this study, we reported the development of a construct using PspCas13b (from *Prevotella* sp.) and human m6A demethylase ALKBH5 catalytic domain. This construct specifically demethylates m6A of targeted mRNAs (WUS, STM, FT, SPL3 and SPL9) to enhance mRNAs stability.

A further improvement of this system could be achieved by integrating **nuclear localization signal (NLS)**, which changes the subcellular location of Cas13aALKBH fusion protein and

enhances the editing efficiency. Meanwhile, the use of full-length ALKBH5 or a more active RNA targeting enzyme such as Cas13b in plants will also greatly improve the efficiency of demethylation. The **dm6ACRISPR** system described here hold great promise to change the game play for future RNA regulation research.

Biotechnological Applications: By elucidating the regulatory roles of m6A modification, this targeted approach could pave the way for biotechnological advancements, such as crop improvement, enhanced stress tolerance, and potentially increased agricultural yields in maize. m6A methylation as a pivotal epitranscriptomic mark in plants, highlighting its significance in post-transcriptional regulation. The demethylation process, catalyzed by specific enzymes known as demethylases or "erasers," such as ALKBH5 and FTO, influences gene expression dynamics linked to root growth and differentiation. These erasers have been found to remove m6A modifications from transcripts associated with root development, thereby impacting root architecture and other essential pathways.

Environmental Stress Responses: Examining the effects of m6A mutations on how plants react to abiotic stressors (such as heat, salt, and drought) will help us understand how plants adjust to changing environmental conditions. This information could be very helpful in creating crop kinds that can withstand stress, which is crucial for the sustainability of agriculture. The m6A methylome is drastically altered by salt stress, which increases m6A modification and mRNA stability of genes linked to salt resistance. These reactions, in turn, positively modulate the tolerance to salt stress.

Socioeconomic Impact: From a socioeconomic standpoint, this research is especially important for nations like Pakistan whose economies heavily rely on agriculture. Crop epigenetic mechanisms can be understood and manipulated to create resilient, high-yielding cultivars that support both economic growth and food security.

Scientists may be able to design maize plants with improved characteristics, such as stress tolerance, increased yield, and greater nutritional value, by using this novel approach. By guaranteeing food security, enhancing livelihoods, and expanding the agricultural sector, such developments in agricultural biotechnology could have significant socioeconomic effects in Pakistan and other agriculturally based economies.

This study makes a significant and innovative addition to agricultural biotechnology and plant genetics. A deeper understanding of gene regulation, stress responses, and crop improvement are made possible by the capacity to precisely control RNA methylation in maize plants. This has significant implications for both scientific research and real-world agricultural applications.

All things considered, the study presents a novel approach to precise RNA modification in maize and provides insights into the complex regulatory systems controlling plant gene expression. Applications for the targeted demethylation system are numerous and range from basic plant biology to possible developments in agricultural biotechnology. This emphasizes the system's importance in comprehending and modifying crop features for better farming methods.

VI. PROJECT DESCRIPTION

Problem Statement: The research challenge lies in the necessity for precise manipulation of mRNA methylation in maize, specifically targeting m6A modifications, to understand their localized impacts on gene expression and biological processes. Current methods lack specificity, causing widespread epigenetic alterations and hindering the study of site-specific RNA modifications in maize. The project team proposes to tackle this challenge by engineering a dCas13b-ALKBH5 fusion protein, named dm6ACRISPR, aimed at enabling locus-specific demethylation of targeted mRNA sequences. This approach will facilitate the elucidation of m6A modification roles in maize gene regulation and cellular processes without inducing broader epigenetic changes.

Existing studies highlight the importance of m6A modifications in RNA metabolism, including mRNA decay, translation efficiency, and stress responses across various organisms. However, in maize, the precise impact of site-specific RNA methylation remains largely unexplored due to the lack of tools allowing targeted modifications. The novel contribution of this research will be the development and application of the dm6ACRISPR system in maize, providing a means to perform precise demethylation at specific mRNA sites. This project aims to fill the existing gap by elucidating the localized effects of m6A modifications on gene expression, mRNA stability, and protein synthesis in maize cells.

The study addresses the pressing need to implement precise mRNA demethylation in maize using an engineered dCas13b-ALKBH5 fusion protein. Current methods for manipulating RNA methylation lack specificity, inducing broad epigenetic alterations, hindering the focused study of site-specific RNA modifications and their consequential impact on gene expression and biological pathways in maize. This investigation aims to fill this gap by developing a targeted RNA demethylation tool, dm6ACRISPR, facilitating locus-specific manipulation of RNA methylation within maize mRNA. The objective is to elucidate the complex functions of m6A alterations in a range of biological processes, such as stress response mechanisms, translation efficiency, and mRNA degradation. In addition to attempting to clarify the precise roles of RNA methylation in maize, this designed technique has potential agricultural uses and provides information that may improve crop attributes and agricultural biotechnology.

In this case, the need to demethylate mRNA in maize using an engineered dCas13b-ALKBH5 fusion protein arises from several reasons:

Specificity in RNA Methylation: The current approaches to modify RNA methylation in maize frequently lead to non-specific, wide-ranging epigenetic alterations. It is difficult to investigate how certain RNA methylation changes affect gene expression and biological processes because of this lack of specificity.

Comprehending Biological Functions: Scientists want to identify the precise functions of m6A RNA methylation in maize. Numerous facets of RNA metabolism, including mRNA decay, translation efficiency, nuclear export, and alternative splicing, have been discovered to be impacted by this alteration. Determining the precise effect of m6A on maize gene expression and development could provide light on crucial biological processes.

Tool Development for Precision Editing: By creating a targeted RNA demethylation tool (dm6ACRISPR), scientists hope to develop a precise method for manipulating RNA methylation at specific sites within maize mRNA. This level of precision allows for the investigation of locus-specific RNA methylation's role in diverse biological processes without inducing broad epigenetic alterations.

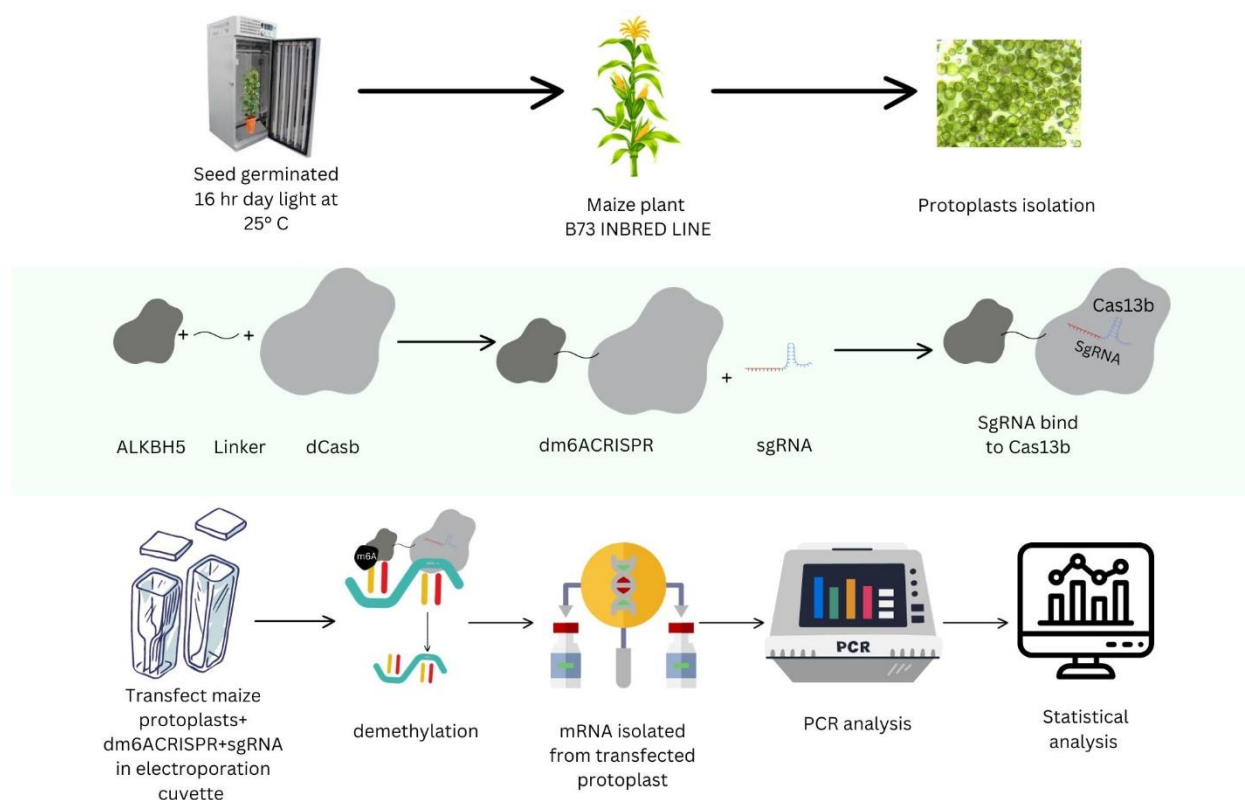
Potential Agricultural Applications: Using targeted approaches to manipulate maize's RNA methylation shows potential for agricultural biotechnology and crop development. Researchers may be able to improve production, stress tolerance, and other desirable features in maize crops by comprehending and managing particular RNA changes.

This research study build upon prior studies that explore RNA modifications and their regulatory roles in plants. Leveraging the CRISPR/Cas system, specifically dCas13b fused with the m6A demethylase ALKBH5, the project extends the capabilities of targeted RNA manipulation into the maize system. By employing this innovative tool, the team seeks to add a comprehensive understanding of the specific impacts of m6A demethylation on maize gene expression and associated biological functions.

In Pakistan's agriculture sector, maize cultivation stands as a vital industry. The application of this research could significantly benefit maize farmers and agricultural biotechnology companies. Using the dm6ACRISPR system to precisely manipulate RNA methylation in maize has the potential to improve yield, overall crop quality, and stress tolerance, among other crop attributes. This development is in line with the goals of the Pakistani maize sector, which is to increase crop productivity and resilience to a range of environmental stressors.

In summary, the goal of employing an engineered dCas13b-ALKBH5 fusion protein to demethylate maize mRNA is to precisely control RNA modifications, comprehend their unique roles in maize biology, and use this knowledge to potentially better agricultural practices.

Graphical Abstract:



Introduction: For more than a century, maize also known as *Zea mays*, has been a mainstay in the genetics and molecular biology research. Its extensive exploration in the genetic mapping, cytogenetics, and epigenetics has been provided valuable insights into regulation of gene expressions important for normal growth, development, and responses to environmental cues in multicellular eukaryotes (1). The intricate orchestration of gene expressions in the maize relies on a complex interplay of the genetic and epigenetic pathways. Maize stands out as an exceptional model for the investigating epigenetic control of the gene expressions due to its substantial genome size, enriched with transposable elements (TEs), and its demonstration of various forms of epigenetic gene regulations. These features offers numerous avenues for detailed mechanistic studies focused on epigenetic phenomenon.

The discovery of RNA chemical modifications originated from observations in the calf liver RNA and has since been recognized as widespread occurrence across all the organisms. Over 160 types of the RNA modifications have been identified, predominantly observed in transfer RNA (tRNA) and ribosomal RNA (rRNA). Among mRNA modifications, the earliest reported one was m6 A, initially detected in mammals and subsequently observed in diverse eukaryotic species, including plants (2). However, technological constraints have hindered rapid progress in comprehending the functional significance of m6 A modifications. Recent breakthroughs involving the identification of m6 A demethylases such as fat mass and obesity-associated protein (FTO) and its homolog a-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) in mammals have reignited biologists' interest in exploring the biological roles of m6 A modifications. m6 A affects almost all aspects of RNA's metabolism, including mRNA decay,

translations efficiency, nuclear retention, nuclear exports, 3' -end processing, and alternative splicing.

The m6A demethylation in maize strongly influences various biological processes, like plant reproductive development, stress response, and gene expression regulation. This highlights its potential in enhancing crop quality and agricultural techniques. Current RNA methylation manipulation methods in maize result in broad epigenetic changes and could activate internal RNA viruses (3). While flavin mononucleotide discovery allows RNA demethylation, understanding specific effects and reducing unwanted outcomes for medical or farming uses remains challenging. Maize-specific RNA methylation targeting is underexplored, leaving a gap in understanding how it impacts diverse biological processes. 'Reader' proteins in maize, like zmETC7, ZmETC8, and ZmETC23, play crucial roles in mRNA processes and protein synthesis. The m6A regulatory mechanisms have been found in multiple plant species and are essential for embryo development, stress response, and fruit ripening in maize. Advances in m6A sequencing demonstrate its vital roles in various plant developmental processes (2, 4).

The CRISPR/Cas system's emergence provides an effective method for exploring genetic functions and variations in nucleic acids, extending beyond human studies to investigate plant systems. Cas13, an RNA-targeting protein, broadens the scope to manipulate RNA dynamics in maize, enabling combat against RNA viruses, common in plant infections. Mutations in Cas13's nuclease domain in maize yield a non-cutting enzyme (dCas13), valuable for studying specific RNA processes. Like dCas9 in human research modifying DNA and histones, recent studies highlight dCas13b-m6A fusion proteins' potential to regulate mRNA in maize. This study aims to create a CRISPR–Cas13b tool to demethylate specific mRNA in maize (5). In this study, we aim to construct and characterize a CRISPR–Cas13b-based tool tailored to target the demethylation of specific mRNA in maize. By fusing the catalytically inactive Type VI-B Cas13 enzyme from *Prevotella* sp. P5–125 (dPspCas13b) with the m6A demethylase ALKBH5 by using a linker, we demonstrate the effectiveness of our engineered construct, named dm⁶ACRISPR, in successfully demethylating targeted mRNA in maize cells. Our construct, dm6ACRISPR, effectively demethylates targeted mRNA, revealing insights into m6A's regulatory impact. Applying dm6ACRISPR to maize mRNA suppresses degradation, showcasing its potential in biotechnology. This work establishes an in vitro tool to dissect mRNA modifications in maize genes and uncover their biological roles.

MATERIAL AND METHODS:

Plant Material and Growth Conditions:

The maize B73 inbred line of *Z. mays* (L.) is used in this study. The seeds were germinated in a growth chamber with a 16-hour light/8-hour dark photoperiod set at 25°C using sterile soil mix (1:1 vermiculite: perlite).

Plasmid Construction:

The dm6ACRISPR system is developed for targeted mRNA demethylation. By overlapping extension PCR and Gibson assembly, the maize-specific m6A demethylase ALKBH5 was fused to dPspCas13b (catalytically inactive Cas13b from *Prevotella* sp. P5–125). The chimeric construct was cloned into a plant expression vector under the control of the maize UBIQUITIN promoter.

Design of Guide RNAs (sgRNAs) for Maize Targets

It should be noted that maize genes have multiple mRNA isoforms; hence an alignment analysis was carried out to check all the mRNA sequences for their isoforms. From conserved regions, sgRNAs were designed that would target specific positions within the 5'UTR, coding sequence (CDS), and 3'UTR of these chosen maize transcripts.

Maize Protoplast Isolation and Transfection:

Isolated maize protoplasts by using a modified procedure of the work of Yoo et al. (2007). The leaves were cut into strips and incubated for 12 to 16 hrs at 28°C in darkness with gentle shaking in enzyme solution containing 1.5% cellulase R-10, 0.75% macerozyme R-10, 0.6 M mannitol, pH 5.7 MES/ DMSO/ NaCl, and BSA (0.1%). Filtration through W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, pH 5.7 MES) was used to wash isolated protoplasts free of cells after the liberation process. The protoplasts were then resuspended in MMG solution at a density of 5×10^5 cells/ml.

For transfection, the electroporation cuvette of diameter equal to 2 mm containing 100 µl of maize protoplasts mixed with these dm6ACRISPR plasmids (10 µg) and sgRNA plasmid (2 µg) was subjected to an electroporation at a voltage of 150V/250µF using Gene Pulser Xcell (Bio-Rad). Transfected protoplasts were incubated at the 25°C for 24 hours (2, 3, 6).

RNA Isolation and m6A Quantification:

The total RNA was extracted from transfected protoplasts by treating them with TRIzol reagent (Thermo Fisher Scientific). After isolating mRNA according to the instructions using a Qiagen mRNA purification kit, an Abcam m6A RNA Methylation Quantification Kit was used for m6A quantification.

RT-qPCR Analysis: The mRNA levels of targeted genes were measured by reverse transcription quantitative PCR (RT-qPCR) using gene-specific primers. A High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) were used for production of cDNA, and SYBR Green Master Mix (Applied Biosystems) was used for qPCR (7). The maize ACTIN gene was used to the normalise the levels of gene expression.

Western Blot Analysis:

The protein extracts from the transfected protoplasts were separated using SDS-PAGE, which was transferred to PVDF membranes and probed with antibodies against housekeeping or m6A-modified proteins. Protein bands were visualized by enhanced chemiluminescence (ECL) detection.

Statistical Analysis: Experiments need at least three biological replicates. Data was analyzed using GraphPad Prism followed by mean \pm standard deviation as indicated. Then, Tukey's post hoc test is done after Student's t-test or one-way ANOVA to determine the significance.

EXPECTED RESULTS:

Design of dm6ACRISPR for Targeted RNA Demethylation in Maize Plants

In the pursuit of developing a tool to investigate RNA modifications in maize plants, we synthesized a hybrid protein. This construct combines the human m6A demethylase ALKBH5 catalytic domain (spanning amino acids 66 to 292) with the inactive form of PspCas13b. We linked these components using an unstructured six-amino-acid sequence (GSGGGG) as a linker, attaching it to either end of the fusion protein. Moreover, we incorporated nuclear localization signal (NLS) peptides at the terminals of both PspCas13b and ALKBH5. The catalytically inactive PspCas13b holds significance as a programmable RNA binding protein (7, 8). Expression of the fused PspCas13b-ALKBH5 sequence was carried out under the control of the CMV promoter, while the CRISPR RNA (gRNA) sequence was placed under the regulation of the maize RNA polymerase III promoter, AtU6. Notably, the conservation of sequences within the CRISPR RNA

might play a crucial role in the activity of Cas13b. Of particular importance, the 3' terminal poly U sequences found in RNA polymerase III-transcribed crRNAs lie in immediate proximity to protospacer sequences involved in RNA recognition. Our investigations revealed the presence of the fusion proteins, both dCas13b-ALKBH5 and ALKBH5-dCas13b, in both the cytoplasm and nucleus of maize cells. This localization was confirmed through immunofluorescence and Western blot analyses. These localization studies underscore the capability of dCas13b-ALKBH5 to access both cellular compartments in maize cells, facilitating precise RNA demethylation at targeted sites.

mRNA stability

In exploring the potential impact of dm6ACRISPR on transcripts harboring multiple m⁶A modifications, the study focused on a maize plant gene analogous to β -catenin, termed ZmCTNNB1. By employing m⁶A-RIP-seq analysis, three potential m⁶A modification sites within the 5'UTR of ZmCTNNB1 were identified: A180 (Site 1, S1), A188 (S2), and A266 (S3). Subsequent m⁶A-RIP-PCR confirmed the presence of m⁶A modifications on ZmCTNNB1 in maize plant cells, confirming the existence of m⁶A methylation. To evaluate the efficacy of dm6ACRISPR, three distinct gRNAs were designed to target the ZmCTNNB1 mRNA, binding either within <150 nt upstream or at a distant location (3042 nt) from the m⁶A sites. The experimental approach involved assessing the impact of these gRNAs on ZmCTNNB1 mRNA expression. Results demonstrated a noticeable reduction in ZmCTNNB1 mRNA expression upon transfection of these gRNAs, in conjunction with wild-type Cas13b, indicating the effectiveness of the gRNAs in maize plant cells. Further analyses unveiled selective demethylation specifically at the S1 and S2 m⁶A sites following the overexpression of ALKBH5 in maize plants. Particularly, gRNA1, targeting a site 125 nt upstream of the S1 m⁶A site, induced an average demethylation at S1 and S2, respectively (9). However, no significant alterations in m⁶A levels were observed at the S3 site using either ALKBH5 overexpression or dm6ACRISPR in maize plants. Consistent with observations in human and mouse cells, dm6ACRISPR in maize plants led to elevated ZmCTNNB1 mRNA levels. Furthermore, dm6ACRISPR increased the mRNA half-life of ZmCTNNB1, indicating augmented mRNA stability in maize plant cells. This enhanced stability might be attributed to reduced binding between ZmCTNNB1 mRNA and its corresponding ZmETC7 protein. Additionally, protein levels analogous to β -catenin exhibited a noticeable increase subsequent to dm6ACRISPR treatment in maize plants. The substantial elevation in protein levels, surpassing the mRNA increase, suggests a potential regulatory role of m⁶A modifications within the 5'UTR of ZmCTNNB1 in translation regulation.

Modulation of translation efficiency in maize

Although it is currently unclear whether m⁶A modification participates in mediating mRNA translation efficiency in maize, several studies in crops obtained affirmative answers on this issue. In maize seedling, transcriptome-wide polysome profiling analysis revealed that m⁶A possesses different effects on translation efficiency, depending on m⁶A abundances and positions in transcripts (9, 10). Concretely, m⁶A modification (demethylation) nearby the start codon or in the 3' UTR with low strength (low m⁶A enrichment value) tends to enhance mRNA translation, while m⁶A with excessive deposition (excessive m⁶A enrichment value) in the 3' UTR decreases the translation efficiency.

mA readers ECT7/ECT8/ECT23 enhance mRNA stability through direct recruitment of the poly(A) binding proteins in maize

RNA N⁶-methyl adenosine (m⁶A) modification is important for plant growth and crop yield. m⁶A reader proteins can be recognized m⁶A modifications to enhance the functions of m⁶A in gene regulation. ECT7, ECT8, and ECT23 are m⁶A readers that are known to redundantly regulate trichome branching and leaf growth, but their molecular functions remain unclear (7, 8). Results Here, we show that ECT7, ECT8, and ECT23 directly interact with each other in the cytoplasm and perform genetically redundant functions in abscisic acid (ABA) response regulation during seed germination and post-germination growth. We reveal that ECT7/ECT8/ECT23 promote the stabilization of their targeted m⁶A-modified mRNAs but have no function in alternative polyadenylation and translation. We find that ECT7 directly interacts with the poly(A) binding proteins, PAB2 and PAB4, and maintains the stabilization of m⁶A-modified mRNAs. Disruption of ECT7/ECT8/ECT23 destabilizes mRNAs of ABA signaling-related genes, thereby promoting the accumulation of ABI5 and leading to ABA hypersensitivity. Our study reveals a unified functional model of m⁶A mediated by m⁶A readers in plants. ECT7/ECT8/ECT23 promote stabilization of their target mRNAs in the cytoplasm.

m6A mRNA Modification in Root Development

m6A mRNA demethylation plays a crucial role in regulating root development in maize, as observed in several research investigating epigenetic modifications in plants. m6A methylation as a pivotal epitranscriptomic mark in plants, highlighting its significance in post-transcriptional regulation. The demethylation process, catalyzed by specific enzymes known as demethylases or "erasers," such as ALKBH5 and FTO, influences gene expression dynamics linked to root growth and differentiation. These erasers have been found to remove m6A modifications from transcripts associated with root development, thereby impacting root architecture and other essential pathways (10). Moreover, studies shed light on the spatial and temporal distribution of m6A demethylation within different zones of maize roots. They highlighted varying demethylation activities across the root tip, elongation zone, and differentiation zone, suggesting that m6A modification dynamics are finely regulated during different stages of root growth.

m6 A mRNA Modification for Crop Improvement

The maize genome has been successfully modified using **dm⁶ACRISPR** technology. Male sterility, lignin biosynthesis, herbicide tolerance, RNA metabolism, secondary metabolism, grain composition, and drought tolerance are among the traits that have been modified using this technique (1). The type of editing used to improve these traits includes template-free gene knockout, targeted editing of a small number of codons using a single-stranded or double-stranded DNA as template, insertion of sequences.

Regulation of the abiotic stresses in crops

In addition to the role in biotic stress, m⁶A also modulates abiotic stress responses in crops. In maize, cadmium treatment induces the differential m⁶A modifications in thousands of transcripts in the root, suggesting that m⁶A may be associated with the abnormal root development caused by cadmium stress. Genes of the m⁶A reader protein ETCs exhibit obvious expression changes in response to abiotic stresses including water and drought stresses. Salt

stress induces a drastic alteration in m⁶A methylome, leading to the increase in m⁶A modification and mRNA stability of genes related to the salt resistance, which in turn positively regulated the tolerance to salt stress (7, 9). Furthermore, significant changes in m⁶A methylome profile, as well as its correlation with mRNA abundance, have been deciphered in pak-choi seedling under heat stress. These results revealed that m⁶A modification also participates in controlling the responses of crops to temperature and humidity-induced stresses. In plants, m⁶A are sensitive to environmental changes. In maize, the m⁶A level decreased significantly upon drought stress, together with the increased expression of potential m⁶A regulators, such as homologs of ALKBH5 and ETC. Notably, in response to drought stress, the m⁶A level decreased in the drought stress-related genes, such as homologs of Actin-7 (ACT7), ECERIFERUM4 (CER4), and CER10. These studies suggest that m⁶A might function during plant responses to environmental stresses, which warrants further investigation.

Project Team, including partnerships:

Targeted mRNA demethylation in maize using a fusion protein of dCAS13b-ALKBH5. This emerging field sits at the intersection of genetic and epigenetic regulation in plants, aiming to manipulate specific RNA modifications for agricultural biotechnology. The proposed team's capacity to deliver results hinges on expertise in several domains. Firstly, molecular biologists with a deep understanding of maize genetics and epigenetics form the backbone. Working together with bioinformaticians proficient in high-throughput sequencing analysis improves their capacity to decipher intricate data. Their research also moves more quickly when they have access to cutting edge facilities like cutting edge molecular biology labs with sequencing and gene editing equipment. Study indicates the importance of m⁶A modifications in RNA, influencing diverse biological processes in plants. Specifically, the fusion of dCAS13b with ALKBH5 presents a novel strategy for precise mRNA demethylation. Studies showcase its potential to modulate mRNA stability, impact translation efficiency, and influence gene expression, thus allowing for specific manipulation without inducing broad epigenetic changes. The team's capacity to deliver timely results stems from their collective expertise, collaborations, and access to advanced facilities. Their goal to decipher the regulatory mechanisms of mRNA modification in maize holds promise for crop improvement and agricultural biotechnology applications.

Year 1:

System Development: The initial phase involves constructing the dm6ACRISPR system by fusing dPspCas13b with the maize-specific m⁶A demethylase ALKBH5. This will be executed using overlap extension PCR and Gibson assembly techniques.

Plasmid Construction: The designed construct will be cloned into a plant expression vector regulated by the maize UBIQUITIN promoter.

Guide RNA Design: Alignment analysis of mRNA sequences for various isoforms will be conducted to design specific guide RNAs targeting regions within the 5'UTR, coding sequence (CDS), and 3'UTR of selected maize transcripts.

Validation: Initial validation and optimization of the dm6ACRISPR system using in vitro assays.

Year 2:

Protoplast Isolation and Transfection: Isolation of maize protoplasts will be performed following a modified protocol. Transfection will be carried out by introducing the constructed dm6ACRISPR plasmid and guide RNA plasmid into the protoplasts using electroporation.

Functional Assessment: Evaluating the efficiency and specificity of the dm6ACRISPR system in demethylating targeted mRNA in transfected maize protoplasts.

Quantification and Analysis: Isolation of RNA from transfected protoplasts and quantification of m6A modifications using specialized kits. Assessing gene expression changes using RT-qPCR analysis.

Year 3:

Protein Analysis: Conducting Western blot analysis to examine m6A-modified proteins in transfected protoplasts.

Optimization and Refinement: Fine-tuning the dm6ACRISPR system based on the findings obtained from previous experiments.

Validation in Plant Systems: Applying the optimized dm6ACRISPR system to maize plants for planta demethylation studies.

Data Compilation and Reporting: Summarizing and compiling all experimental data and findings for publication in scientific journals and presentations at relevant conferences.

Milestones and Deliverables:

Constructed dm6ACRISPR system: Year 1

Validated dm6ACRISPR system in vitro: End of Year 1

Efficient demethylation in maize protoplasts: End of Year 2

Optimized dm6ACRISPR system: Mid-Year 3

In planta demethylation studies in maize plants: End of Year 3

Publication of findings in scientific journals: After completion of experiments

Presentation of results at conferences: Throughout the project timeline, as appropriate.

Ethical considerations related to the proposed research (If any):

Certainly, in the realm of targeted mRNA demethylation research, several ethical considerations warrant attention. Here are some aspects that could raise ethical concerns:

Unintended Effects: Modifying mRNA methylation may result in unexpected modifications to gene expression, which could impact the characteristics of the plant or modify how it interacts with its surroundings.

Environmental Impact: Changes to the plants may have an impact on the local flora and wildlife, which could have ecological ramifications. Even though the main emphasis of this study is plant genetics, it's crucial to include any possible environmental effects. Modifying RNA or changing gene expression in plants may have unanticipated effects on the ecology. Any field testing or environmental release of genetically modified plants should be preceded by a comprehensive risk assessment.

Informed Consent: Involvement of research participants, in this case, could refer to those involved in the study, handling of genetic materials, or potential environmental interactions. Ensuring informed consent regarding the handling of genetic materials or potential environmental impact is crucial. Given this research involves experimentation on plants (maize), informed consent isn't a direct concern as it would be in human studies. However, it's essential

to highlight that ethical research involves transparency, even with plant-based experiments. This includes clear communication about the experimental process, potential impacts on plant growth or health, and the purpose of the study.

Responsible Use of technology: Although CRISPR-based technologies have a lot of potential, their proper usage raises ethical concerns. Crucial ethical considerations include making sure genetically modified organisms are properly contained, preventing unintentionally spread, and weighing the effects of releasing modified organisms into the wild.

To address these concerns, the Principal Investigator plans to:

Risk Assessment: Conduct thorough risk assessments to anticipate potential unintended consequences of manipulating mRNA methylation. Precautionary measures will be implemented to minimize any unforeseen effects.

Environmental Monitoring: Implement stringent monitoring protocols to evaluate any environmental impact. This includes studying the plants in controlled settings before any potential release into the environment.

Stakeholder Engagement: Communicate honestly about the research, its consequences, and any concerns you may have with experts, regulatory agencies, and communities that may be impacted.

Informed Consent Procedure: A thorough informed consent procedure will be adhered to for any participation involving people or participants. This entails getting the express consent of all people involved and outlining the nature of the study, goals, and possible dangers.

The Principal Investigator will ensure compliance with ethical guidelines, involving an institutional review board or ethics committee to oversee the research ethical aspects. This committee will be responsible for evaluating and approve the ethical conduct of the study,

VIII. IMPLEMENTATION TIMELINE

For instance, in the context of the targeted mRNA demethylation project in maize using the engineered dCAS13b-ALKBH5 fusion protein, the implementation timeline might look something like this:

Preparation Phase:

Initiation and Proposal Development (Months 1-2): This include planning the project's design, establishing its parameters, and creating a thorough research proposal. During these months, preliminary planning, budgeting, and resource identification take place.

Team Formation and Roles Defined (Month 2): Following approval of the proposal, the research team is put together, with each member's precise duties and responsibilities laid out to guarantee a smooth transition.

Resource Procurement and Lab Setup (Months 3-4): The essential tools, supplies, and materials are acquired during this stage. Set up, calibrated, and prepared for experimentation are laboratories.

Research and Development Phase:

Design and Construction of Fusion Protein (Months 5-7): The team works on designing and creating fusion protein, involving molecular biology techniques and genetic engineering to merge the inactive Cas13b with the ALKBH5 demethylase.

Guide RNA Design and Optimization (Months 6-8): It is essential to design the guide RNAs (sgRNAs). The group finds conserved areas in the transcripts of the maize genes and refines sgRNAs to target particular mRNA sections.

Protoplast Isolation and Transfection Protocol Establishment (Months 9-10): A dependable transfection technique is established and a methodology for isolating maize protoplasts is designed.

Validation of dm6ACRISPR System In Vitro (Months 11-12): Initial validation experiments are conducted in controlled lab conditions to assess the functionality and effectiveness of the engineered system.

Experimental Phase:

Maize Protoplast Isolation and Transfection (Months 13-15): During these months, actual experiments are conducted to isolate maize protoplasts and transfect them with the designed dm6ACRISPR system.

RNA Isolation and m6A Quantification (Months 16-17): The transfected protoplasts' RNA is extracted, and specific tools and methods are used to measure the amounts of m6A modification.

RT-qPCR Analysis and Western Blot Studies (Months 18-20): The team performs gene expression analyses using RT-qPCR and assesses protein levels through Western blotting to study the effects of the demethylation system.

Data Analysis and Interpretation:

Analysis of mRNA Stability and m6A Modification (Months 21-22): Collected data from experiments are rigorously analyzed to understand the impact of targeted demethylation on mRNA stability and modification levels.

Interpretation of Results and Comparative Studies (Months 23-24): Results are interpreted, compared with existing literature or control groups, and conclusions are drawn to understand the implications of the findings.

Publication and Documentation:

Manuscript Writing and Review Process (Months 25-27): The research results are combined into a paper, which is then carefully reviewed by peers to ensure correctness and validity.

Submission to Peer-Reviewed Journals (Months 28-30): During months 28 and 30, the completed article is sent for publication to scholarly journals.

Documentation of Experimental Protocols and Findings (Months 31-32): For future use and distribution, thorough documentation of the experimental protocols, procedures, and results is generated.

Dissemination and Future Prospects:

Presentation at Conferences or Scientific Gatherings (Months 33-34): To exchange ideas and get input from the scientific community, the team presents their findings at conferences or seminars.

Sharing Findings with Agricultural Biotech Industry (Months 35-36) The project's prospective uses in agricultural biotechnology are emphasised, and conversations or partnerships with business partners are started for real-world applications.

YEAR ONE	
Major Tasks	Deliverables
<ul style="list-style-type: none"> • System Development 	Constructed dm6ACRISPR system
<ul style="list-style-type: none"> • Plasmid Construction 	Cloned construct into plant expression vector
<ul style="list-style-type: none"> • Guide RNA Design 	Designed specific guide RNAs for maize targets
<ul style="list-style-type: none"> • Initial Validation 	Validation and optimization in vitro
<ul style="list-style-type: none"> • Optimization 	Fine-tuning the dm6ACRISPR system

YEAR TWO	
Major Tasks	Deliverables
<ul style="list-style-type: none"> • Protoplast Isolation & Transfection 	Isolated maize protoplasts and transfection
<ul style="list-style-type: none"> • Functional Assessment 	Evaluated dm6ACRISPR system efficiency
<ul style="list-style-type: none"> • Quantification & Analysis 	Quantified m6A modifications and gene expression
<ul style="list-style-type: none"> • Protein Analysis 	Conducted Western blot for m6A-modified proteins
<ul style="list-style-type: none"> • System Refinement 	Optimized dm6ACRISPR based on findings

YEAR THREE	
Major Tasks	Deliverables
<ul style="list-style-type: none"> • Validation in Plant Systems • Data Compilation • In planta Demethylation • Finalization & Reporting • Presentation Preparation 	<ul style="list-style-type: none"> Applied dm6ACRISPR to maize plants Compiled experimental data and findings Conducted in planta demethylation studies Summarized findings for publication and reporting Prepared presentations for conferences

IX. PHYSICAL RESOURCES AND FACILITIES

The laboratory instruments needed for conducting experiments involving targeted mRNA demethylation in maize using an engineered dCAS13b-ALKBH5 fusion protein:

PCR Machine: This apparatus is fundamental for Polymerase Chain Reaction (PCR), employed to amplify specific DNA sequences essential for constructing the dm6ACRISPR system. It allows the replication of targeted DNA segments via thermal cycling.

Gel Electrophoresis System: Used for separating and analyzing DNA fragments produced during molecular processes. The system includes gel tanks, power supplies, and imaging systems. It enables the visualization and analysis of DNA fragments to verify successful amplification or isolation.

Centrifuge: This apparatus is essential for separating individual cells and removing impurities from DNA/RNA samples. Based on their density, it separates various components by rapidly spinning samples.

Biosafety Cabinet: A biosafety cabinet reduces the risk of contamination by offering a clean, safe workspace for handling genetic materials and performing molecular biology processes.

Incubation Area at 28°C and Shaking Incubators: Shaking incubators and an incubator set at 28°C are used for the enzymatic leaf incubation process, which isolates protoplasts from leaves. They facilitate the release of protoplasts from the plant material by keeping the temperature at a constant 28°C and agitating the mixture gently.

Electroporation Equipment (Electroporation Cuvettes, Gene Pulser Xcell): Equipment for electroporation (Electroporation Cuvettes, Gene Pulser Xcell): Required to transfect maize protoplasts with plasmids that have been created. Throughout the procedure, the sample is held in place by electroporation cuvettes while electrical pulses are delivered to the protoplasts by the Gene Pulser Xcell.

RNA Extraction and Purification Kits (TRIzol Reagent, mRNA Purification Kit):

These kits are essential for purifying particular mRNA and separating total RNA from transfected protoplasts. The mRNA purification kit separates the mRNA from the total RNA sample, whereas the TRIzol reagent is utilised for the first round of RNA extraction.

m6A RNA Methylation Quantification Kit: Using this specialised kit, one may measure the amount of m6A RNA that has been methylated, which allows one to determine the status of RNA modification.

Thermal Cycler: For reverse transcription and quantitative PCR (RT-qPCR) in gene expression studies, a thermal cycler is necessary. It makes the temperature cycling required for measurement and amplification of DNA easier.

SDS-PAGE Setup and PVDF Membranes: PVDF Membranes and SDS-PAGE Setup: These are used to separate proteins by gel electrophoresis and then transfer them to PVDF membranes for Western blot analysis.

Enhanced Chemiluminescence (ECL) Detection System: Use in Western blot analysis for visualize protein band. It utilizes chemiluminescent reactions to detected specific proteins on PVDF membranes.

Computational Resources: These include computers equipped with software for sequence alignment, primer design, and statistical analysis. They are used for data processing and analysis.

In the context of our existing lab setup, several crucial pieces of equipment are already available, serving as the foundation for conducting genetic experiments. The PCR machine stands as a fundamental tool, facilitating DNA amplification, particularly vital in constructing genetic systems like the dm6ACRISPR system. An incubator provides a controlled environment for enzymatic leaf

treatments during maize protoplast isolation, ensuring consistent temperatures for effective results. Additionally, the electroporator plays a pivotal role in transfecting genetic material into maize protoplasts, enabling the introduction of foreign DNA into these cells. The GE electrophoresis system aids in the analysis and visualization of DNA fragments, crucial for verifying DNA manipulation success, while the centrifuge serves in isolating cellular components and purifying DNA/RNA samples, an essential step in many molecular procedures. The water bath, UV illuminator, and biosafety cabinet further contribute to maintaining specific conditions, visualizing DNA fragments, and providing a sterile workspace, respectively.

However, considering the experiment's complexity and the missing components in your lab, several pieces of equipment could substantially enhance the experiment's efficiency and depth. Enzyme solution preparation equipment is pivotal for consistent enzymatic leaf treatments during protoplast isolation, ensuring precise and effective results. Access to computational resources, including computers equipped with essential software for sequence analysis, primer design, and statistical analysis, would expedite data processing and interpretation. Obtaining a customised m6A RNA Methylation Quantification Kit is essential since it allows accurate measurement of m6A RNA methylation levels, which is an important component of the experiment. In addition, reverse transcription and quantitative PCR (RT-qPCR) for the measurement of gene expression require a thermal cycler, and RNA-related processes would be more efficiently handled with an RNA isolation/purification kit specifically designed for this purpose.

Considering the equipment that is still missing is essential to the effective completion of our research, I would like to submit a budget request to improve our laboratory's capabilities. The following items are essential to bolster our research endeavors:

Enzyme Solution Preparation Equipment: Acquiring accurate volume measurement tools and mixing apparatus is essential to guaranteeing reliable and efficient enzymatic leaf treatments during maize protoplast isolation. Cost estimate: \$1000.

Computational Resources: For effective data processing and interpretation, having access to computers with the required software for statistical analysis, primer creation, and sequence analysis is essential. Cost estimate: \$1500.

m6A RNA Methylation Quantification Kit: An essential component of our investigation, this specialised kit allows precise measurement of m6A RNA methylation levels. Cost estimate: \$2200.

Thermal Cycler: Essential for quantitative PCR (RT-qPCR) and reverse transcription in the study of gene expression. Cost estimate: \$1300.

RNA Isolation/Purification Kit: A specialised kit designed for RNA purification and extraction will greatly improve and expedite RNA-related processes. Cost estimate: \$2000.

X. PROJECT WEAKNESSES AT LAUNCH

Potential weaknesses and strategies to overcome them for your project:

Technological Limitations: The project might face challenges due to the novelty and complexity of the technology involved in modifying RNA methylation in maize. To overcome this, extensive validation and optimization of the dm6ACRISPR system, particularly its specificity and efficiency, could be carried out in a stepwise manner. Additionally, collaborations with experts in CRISPR technology or epigenetics can provide valuable insights and guidance.

Target Specificity: Ensuring precise targeting of specific mRNA regions for demethylation is crucial. There might be difficulties in achieving absolute target specificity, potentially causing off-target effects. To address this, rigorous computational predictions combined with empirical testing of guide RNA designs should be implemented. Continuous improvement of bioinformatics tools for sgRNA design and regular assessment for off-target effects are essential.

Delivery System Efficiency: The effective delivery of the dm6ACRISPR system into maize cells, especially considering the complex cell wall structure, could pose challenges. Strategies involving novel delivery systems like nanoparticles or viral vectors tailored for plant cells could be explored. Enhancing system delivery can be achieved by developing more efficient delivery vehicles and optimising transformation methods.

Regulatory and Ethical Considerations: Given that genetically modified organisms will be released into the environment, there may be regulatory obstacles or ethical issues that the research must address. It is essential to interact with regulatory agencies, follow biosafety protocols, and carry out in-depth risk assessments. In order to assure responsible research techniques and navigate ethical considerations, collaborations with ethicists, policymakers, and stakeholders might be beneficial.

Functional Characterization: It may be difficult to comprehend the wider effects of targeted RNA demethylation on the physiology and gene expression of maize. It will be essential to carry out in-depth research on how RNA demethylation affects plant growth, development, and stress responses downstream. Multi-omics techniques and long-term research can offer a comprehensive knowledge.

XI. RISK MANAGEMENT STRATEGY

The proposed project involving targeted mRNA demethylation in maize using an engineered dCAS13b-ALKBH5 fusion protein face several potential risk that can impact its progress and success. One significant risk involves the efficacy and specificity of the engineered tool itself. To address this, an initial strategy would focus on comprehensive in vitro testing to ensure the accuracy and efficiency of the dm⁶ACRISPR system in demethylating targeted mRNA in maize cells. This phase would include meticulous validation and optimization protocols, aiming to minimize off-target effects and enhance precision.

Another considerable risk involves regulatory challenges or ethical concerns associated with genetic modification in plants. To manage the risk, the project would adhered strictly to regulatory instructions and ethical framework governing genetic modification in plants. Collaboration with regulatory bodies and ethical review board will be established to ensure compliance with established standard and guideline.

Furthermore, technological limitations or unexpected technical hurdles could impede the project's progress. To mitigate this risk, a contingency plan involving ongoing technology assessment and adaptation would be put in place. Regular evaluation of emerging technologies or modifications to the dm⁶ACRISPR system would occur to address any unexpected limitations. To address unanticipated risks that may arise throughout the project's duration, a proactive approach that incorporates ongoing monitoring and assessment would be implemented. Frequent checkpoints and progress reviews would make it possible to spot any new hazards or departures from the expected results early on. This methodology allows for quick adjustments or changes to the research design, keeping the project on track to meet its objectives.

Furthermore, a key component of risk management would be to promote an interdisciplinary and collaborative approach. It would be easier to identify and address any unforeseen risks quickly if the project team, institutional leadership, and partners were to foster open communication and insight sharing.

To ensure the successful execution of the research while mitigating potential risks, the project's risk management strategy ultimately entails a proactive, multifaceted approach that integrates thorough testing, compliance with regulations, adaptability to technological advancements, continuous monitoring, and collaborative engagement among stakeholders.

There are a number of possible hazards associated with the project that could prevent it from moving forward and succeeding. These risks may include:

Technical Challenges: Implementing the dm⁶ACRISPR system in maize cells may encounter technical hurdles, such as inefficient transfection methods or variability in RNA demethylation efficiency across different targets.

Off-target Effects: There might be unintended effects on non-targeted mRNA or unintended modifications due to the specificity of the dCas13b-ALKBH5 fusion protein.

Regulatory and Ethical Considerations: Regulatory hurdles concerning the use of genetic manipulation techniques in plants could pose challenges. Additionally, ethical considerations regarding the potential environmental impact or unintended consequences need addressing.

The strategy to mitigate these risks involves proactive measures:

Comprehensive Planning: Before the project begins, the Principal Investigator (PI) will supervise careful planning, guaranteeing comprehensive feasibility studies and technical

evaluations. Diverse viewpoints and experience in risk assessment will be provided by collaborators and institutional leadership.

Quality Controls and Validation: Rigorous testing protocols will be implemented to validate the efficacy and specificity of the dm6ACRISPR system. Regular quality checks and control experiments will help in early detection of technical issues.

Constant Monitoring and Adaptation: The project team will keep a close eye on developments and quickly employ adaptable solutions to tackle any technological obstacles. Progress reports and regular team meetings will help make necessary adjustments on time.

Concerning unanticipated hazards that could appear throughout the course of the project:

Dynamic Risk Assessment: Periodically, the project team, comprising the collaborators, institutional leadership, and principal investigator, will carry out risk assessments. This will entail reevaluating risks in a dynamic project context, analysing continuing data, and identifying any developing difficulties.

Adaptive Response Mechanisms: To quickly address unanticipated hazards, an agile methodology will be used. Plans for contingencies will be implemented so that research protocols and methodologies can be quickly adjusted as needed.

Collaborative Decision-Making: Collaborative Decision-Making: To address emerging risks, the PI will work closely with collaborators and institutional leadership to make well-informed decisions. Establishing regular channels of communication will speed up the sharing of knowledge and the creation of consensus.

To put risk management plans into action, the Principal Investigator will closely coordinate with collaborators and institutional leadership. Regular meetings, honest communication, and group decision-making will all be part of this partnership to successfully manage risks and make sure the project is successful. Throughout the course of the project, transparency and compliance with regulatory requirements will also be given top priority.

XIII. LIST OF REFERENCES

1. Shao Y, Wong CE, Shen L, Yu H. N6-methyladenosine modification underlies messenger RNA metabolism and plant development. *Current Opinion in Plant Biology*. 2021;63:102047.
2. Shi H, Xu Y, Tian N, Yang M, Liang F-S. Inducible and reversible RNA N6-methyladenosine editing. *Nature Communications*. 2022;13(1):1958.
3. Stepper P, Kungulovski G, Jurkowska RZ, Chandra T, Krueger F, Reinhardt R, et al. Efficient targeted DNA methylation with chimeric dCas9–Dnmt3a–Dnmt3L methyltransferase. *Nucleic acids research*. 2017;45(4):1703-13.
4. Sun X, Wang DO, Wang J. Targeted manipulation of m6A RNA modification through CRISPR-Cas-based strategies. *Methods*. 2022;203:56-61.
5. Wei J, He C. Site-specific m6A editing. *Nature chemical biology*. 2019;15(9):848-9.
6. Xu X, Tao Y, Gao X, Zhang L, Li X, Zou W, et al. A CRISPR-based approach for targeted DNA demethylation. *Cell discovery*. 2016;2(1):1-12.
7. Liang Z, Riaz A, Chachar S, Ding Y, Du H, Gu X. Epigenetic modifications of mRNA and DNA in plants. *Molecular Plant*. 2020;13(1):14-30.
8. Diez CM, Roessler K, Gaut BS. Epigenetics and plant genome evolution. *Current opinion in plant biology*. 2014;18:1-8.
9. Du X, Fang T, Liu Y, Wang M, Zang M, Huang L, et al. Global profiling of N6-methyladenosine methylation in maize callus induction. *Plant Genome* 13: e20018. 2020.
10. Liu X-M, Zhou J, Mao Y, Ji Q, Qian S-B. Programmable RNA N 6-methyladenosine editing by CRISPR-Cas9 conjugates. *Nature chemical biology*. 2019;15(9):865-71.

XIV. PROPOSED PROJECT BUDGET

XV. REQUIRED ATTACHMENTS

1. Letter of Support (maximum two pages) from the University Head (Vice-Chancellor/ Rector). The letter should: confirm the institutional commitment to the proposed project.
2. Letter of Support from Head of the Collaborating Institutions. The letter should: confirm the institutional commitment to the proposed project.
3. Affidavit for time commitment and honorarium of PI and Co-PIs by the respective Head of the (University (VC/ Rector) and the collaborating Institutions/ organizations)
4. Appointment letter from the PI & Co-PIs to confirm their affiliation with Universities and collaborating Institutions
5. Last pay slip of Pakistani PI and Co-PIs for finalizing the personnel cost in Budget
6. Letters of Commitment (maximum two pages each)
 - From local or sectoral collaborators (maximum 3 letters).
 - From academic collaborators (maximum 3 letters)
 - The letters should describe how the collaborator will contribute to the proposed project goals. The letter should also describe how the collaborator will support their costs associated with participating in the project.
7. Ethical Certificate duly signed by Ethical Research Committee of Institute
8. Signed and dated Curriculum Vitae of the following individuals:
 - Principal Investigator and CoPIs
 - Up to five additional academic, local or sectoral collaborators who will contribute to the proposed project
 - The CVs should be of a standard form (maximum two pages) and include the following; information:
 - Full Name
 - Position/Title
 - Institution
 - Professional Training/Education
 - Chronological List of Positions
 - List of up to five publications related to the proposed project, in standard citation format
 - List of up to five activities related to the proposed project. These activities may include: current or previous grants; teaching; collaborations; leading workshops/conferences; community outreach or engagement; consulting; etc.

Declaration Certificate:

It is hereby certified that:

- a) PI is a full time regular faculty member of HEI or if is hired on contract, same is not less than project life/duration.
- b) The university will spare the faculty members from any teaching or administrative responsibilities against their time committed on the proposed project.
- c) Equipment(s) demanded for the proposed project is / are not available in the University / Institute.
- d) No portion of the proposed project has been submitted and /or funded by HEC or any other funding agency.
- e) The proposed project is genuinely novel and that there is no plagiarized material including self-plagiarism.
- f) PI has never been blacklisted by HEC.
- g) PI is not executing any other project of HEC which is delayed.
- h) Decision of HEC will be considered final and will not be challenged in a court of law.
- i) The University/DAI will provide complete support and facilitation to the PI and his project team for the establishment & operation of the proposed project, if approved by HEC and funds awarded to the University/ DAI. Accordingly, the University/ DAI will provide necessary facilities for smooth execution of the project including land, building, space, laboratories, machinery, equipment, transport, amenities like utilities and other services.
- j) The University/DAI will get clearance from HEC (Project Completion Certificate /Project Clearance Certificate (PCC)) in order to relieve the PI, for any reason e.g. for postdoc leave/EOL/study leave/ termination of job etc, if the proposed project is awarded by HEC.
- k) The University/DAI will not replace the PI of the proposed project without getting prior permission from HEC in writing.

Signature of Principal Investigator

Name:

Designation:

Department:

University/ DAI Name

**Signature with Stamp of Director
(ORIC/Research Office)**

Name:

Designation:

University/DAI Name

**Signature with Stamp of the Head of University/ DAI
(Vice-chancellor/Rector)**

Name:

University/ DAI Name: