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In silico Design of sgRNA for CRISPR/Cas9-Mediated MLO Gene Mutagenesis to Reduce Powdery Mildew Susceptibility in *Vitis vinifera*

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Abstract:** Powdery mildew (Erysiphe necator) poses a severe threat to Vitis vinifera (grapevine) cultivation worldwide, causing substantial yield and quality losses. Conventional fungicide-based management raises sustainability concerns and risks of pathogen resistance. The Mildew Locus O (MLO) gene family, which encodes transmembrane proteins functioning as negative regulators of plant immunity, represents a compelling target for durable disease resistance through loss-of-function mutagenesis. This study presents a comprehensive in silico workflow for the design and evaluation of single-guide RNAs (sgRNAs) for CRISPR/Cas9-mediated mutagenesis of VvMLO7 (Accession: EU591726) in grapevine. Using the CHOPCHOP v3 tool, 18 candidate sgRNAs were generated and filtered based on GC content (35–65%), self-complementarity, predicted on-target efficiency, and off-target mismatch scores. Two optimal sgRNAs were selected: sgRNA1 (GTGGGGACACACCATTCGAGTGG; efficiency 65.95%) and sgRNA2 (TCCCGTGTTCACTTATTGCAAGG; efficiency 59.05%). Secondary structure analysis via RNAfold confirmed canonical stem-loop conformations with minimum free energies of −28.60 and −27.40 kcal/mol, respectively. Both sgRNAs were integrated into a pCas9-TPC plant-compatible vector incorporating homology-directed repair (HDR) donor sequences and a GFP selection marker. This in silico framework provides a rational, cost-effective foundation for experimental validation and advances precision breeding approaches for sustainable viticulture.
Keywords:** CRISPR/Cas9; VvMLO7; sgRNA design; powdery mildew; Vitis vinifera; genome editing; in silico; plant immunity.

I. INTRODUCTION

Vitis vinifera (grapevine) is among the most economically significant fruit crops globally, cultivated extensively for fresh consumption and wine production. Powdery mildew, caused by the obligate biotrophic fungus Erysiphe necator, is one of the most destructive diseases affecting grapevine cultivation. The pathogen thrives under warm, dry conditions (18–22°C) and causes severe yield losses, fruit quality deterioration, and reduced marketability [1]. The economic impact of powdery mildew is further exacerbated by the heavy reliance on chemical fungicides, which raise environmental concerns and risk the emergence of fungicide-resistant pathogen strains [2].

A key determinant of powdery mildew susceptibility in plants is the Mildew Locus O (MLO) gene family, which encodes a class of transmembrane proteins that function as negative regulators of plant immunity [3]. Loss-of-function mutations in MLO genes confer broad-spectrum, durable resistance to powdery mildew across multiple plant species, including barley, Arabidopsis thaliana, tomato, and grapevine. This phenomenon has been well-documented since the initial discovery of naturally occurring mlo mutants in barley [4].

The advent of CRISPR/Cas9 genome editing technology has transformed crop improvement by enabling precise, heritable, and multiplexable modifications at specific genomic loci [5]. The system employs a single-guide RNA (sgRNA) to direct the Cas9 endonuclease to the target site, where it introduces a double-strand break (DSB). Subsequent repair via error-prone non-homologous end joining (NHEJ) or template-directed homology-directed repair (HDR) produces targeted insertions, deletions, or defined sequence changes [6].

In silico sgRNA design is a critical prerequisite for effective CRISPR/Cas9 editing. Computational tools allow researchers to rationally select guide sequences with high on-target activity, minimal off-target potential, and favorable secondary structures before proceeding to experimental validation [7]. This study presents a systematic in silico pipeline for designing, evaluating, and constructing a CRISPR/Cas9-based system for targeted mutagenesis of the VvMLO7 gene in Vitis vinifera, with the overarching goal of engineering durable resistance to powdery mildew.

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II. BACKGROUND

A. Powdery Mildew in Grapevine

Powdery mildew caused by Erysiphe necator is a biotrophic pathogen that infects leaves, shoots, flowers, and berries of grapevine. The disease is characterized by white, powdery fungal growth on infected tissues. On leaves, early signs manifest as small white spots that expand, causing distortion, reduced photosynthesis, and necrosis. Infected shoots may become stunted or twisted. Berries may shrivel, crack, or prematurely ripen, compromising fruit sugar content and wine quality. Flower clusters suffer poor fruit set, reducing overall yield [8]. The life cycle of E. necator involves both asexual (anamorphic) and sexual (teleomorphic) phases. The fungus overwinters as cleistothecia on dormant plant tissue and produces airborne conidia in spring that initiate new infections. The sexual phase involves the formation of cleistothecia under unfavorable late-season conditions, producing ascospores capable of overwintering for subsequent seasons. The pathogen does not require free moisture for infection, making it particularly problematic in Mediterranean-type climates with warm, dry summers and moderate humidity [9].

B. The MLO Gene Family and Powdery Mildew Susceptibility

The MLO gene family encodes seven-transmembrane domain proteins localized to the plasma membrane, where they act as negative regulators of defense responses. In their normal physiological role, MLO proteins suppress a form of cell death (hypersensitive response) that would otherwise restrict pathogen spread. Loss of MLO function de-represses this immune pathway, conferring what is known as 'mlo resistance' — a recessive, broad-spectrum, and durable form of resistance. First characterized in barley (Hordeum vulgare) through spontaneous mlo mutants, this resistance has since been identified in Arabidopsis, tomato, cucumber, pea, and now grapevine [4, 10]. In Vitis vinifera, several MLO paralogs have been characterized through expression profiling. Among these, VvMLO3, VvMLO4, and VvMLO17 have emerged as primary contributors to powdery mildew susceptibility, with VvMLO7 also implicated based on its expression patterns and domain conservation [3]. Targeted disruption of these susceptibility genes via CRISPR/Cas9 offers a route to engineering mlo-type resistance in grapevine cultivars that currently lack natural resistance.

C. CRISPR/Cas9 in Grapevine Research

Despite grapevine's recalcitrance to genetic transformation and its woody perennial nature, successful CRISPR/Cas9 editing studies have been reported. Malnoy et al. (2016) demonstrated transgene-free editing of V. vinifera protoplasts using Cas9 ribonucleoproteins (RNPs), targeting the PDS and WRKY genes [11]. Stable integration has been achieved using Agrobacterium-mediated transformation, though concerns about transgene retention in edited lines have motivated the development of transgene-free approaches. Ongoing challenges include low transformation efficiency, tissue culture-dependent regeneration, and the need for multiplexed editing to address MLO gene redundancy.

III. MATERIALS AND METHODS

A. Sequence Retrieval and Analysis

The nucleotide sequence of the VvMLO7 gene was retrieved from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) using the accession number EU591726. The sequence represents a partial mRNA coding sequence and was selected based on its functional annotation, domain conservation, and established relevance to powdery mildew susceptibility. The retrieved FASTA sequence was analyzed for open reading frame (ORF) structure, conserved transmembrane domains, and calmodulin-binding motifs characteristic of MLO family proteins. The sequence served as the input template for all downstream in silico analyses.

B. In Silico sgRNA Design

Single-guide RNA sequences targeting VvMLO7 were designed using CHOPCHOP v3 (https://chopchop.cbu.uib.no/), a widely used web-based tool supporting plant genome editing applications [12]. The VvMLO7 coding sequence (EU591726) was submitted as the target input using the SpCas9 system with an NGG PAM requirement. Candidate sgRNAs were evaluated and filtered based on the following criteria:

- GC content between 35% and 65% to ensure optimal binding thermodynamics
- Self-complementarity score of 0 to minimize risk of intramolecular RNA folding
- Predicted on-target editing efficiency of ≥40%
- Mismatch scores (MM0–MM3) reflecting off-target binding potential across the Vitis vinifera genome
- Target location within early exons to maximize the probability of frameshift mutations

Two sgRNAs meeting all selection thresholds were chosen for downstream structural analysis and vector integration.

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C. RNA Secondary Structure Prediction

The secondary structures of the two selected sgRNAs were predicted using the RNAfold Web Server (http://rna.tbi.univie.ac.at/) from the ViennaRNA Package 2.0 [13]. Each sgRNA spacer sequence was appended with a standard S. pyogenes Cas9 scaffold sequence, and the resulting full-length guide RNA was submitted for minimum free energy (MFE) structure prediction. Structural parameters assessed included number of stem-loops, hairpin loops, internal bulges, length of the longest unbranched stem, number of unpaired bases in the guide region, and overall MFE (kcal/mol).

D. In Silico CRISPR/Cas9 Vector Design

A plant-compatible CRISPR/Cas9 expression vector was designed in silico using A Plasmid Editor (ApE) software. The pCas9-TPC backbone, which expresses Cas9 under a plant-compatible promoter, was selected as the base vector. The construct was modified to support HDR-based knock-in through the inclusion of:

- Two sgRNA expression cassettes under the AtU6-26 promoter. sgRNA1 was inserted at MluI and PacI restriction sites; sgRNA2 was cloned at SpeI and XbaI sites.
- A donor DNA repair template flanked by 500–800 bp homology arms corresponding to genomic sequences upstream and downstream of the Cas9 cleavage sites within VvMLO7.
- A GFP reporter gene inserted at the SmaI site to enable visual screening of transformed plant cells.

IV. RESULTS

A. Sequence Retrieval and Analysis of VvMLO7

The VvMLO7 gene sequence (Accession: EU591726) was successfully retrieved from NCBI GenBank. The partial mRNA coding sequence was confirmed to contain hallmark features of the MLO gene family, including predicted transmembrane helices and calmodulin-binding domains, consistent with its function as a susceptibility (S) gene. The sequence was validated by cross-referencing with the annotated Vitis vinifera genome, and no sequence artifacts or annotation discrepancies were detected. These conserved structural features confirm VvMLO7's role as a negative regulator of plant immunity and justify its selection as a CRISPR/Cas9 editing target.

B. sgRNA Design and Evaluation

CHOPCHOP v3 identified 18 candidate sgRNAs targeting the VvMLO7 coding sequence. After applying the defined selection criteria, two sgRNAs were identified as optimal candidates (Table 1). sgRNA1 (GTGGGGACACACCATTCGAGTGG), located at position 45 on the negative strand, exhibited a GC content of 60%, a self-complementarity score of 0, and a predicted on-target efficiency of 65.95%. sgRNA2 (TCCCGTGTTCACTTATTGCAAGG), located at position 103 on the positive strand, had a GC content of 45%, a self-complementarity score of 0, and a predicted efficiency of 59.05%. Both sgRNAs demonstrated low mismatch scores across MM1–MM3, indicating minimal off-target binding potential in the V. vinifera genome.

Table 1: Selected sgRNAs for VvMLO7 CRISPR/Cas9-mediated mutagenesis

Rank	Target Sequence	Genomic Location	Strand	GC Content (%)	Self-comp.	MM1	MM2	Efficiency (%)
1	GTGGGGACACACCATTCGAGTGG	EU591726.1:45	−	60	0	0	0	65.95
11	TCCCGTGTTCACTTATTGCAAGG	EU591726.1:103	+	45	0	0	0	59.05

MM1/MM2: off-target mismatch scores at 1 and 2 positions; Self-comp.: self-complementarity score

C. RNA Secondary Structure Prediction

RNAfold analysis of both sgRNAs confirmed the formation of stable secondary structures characteristic of functional CRISPR guide RNAs. Both sequences folded into canonical stem-loop conformations with dense, well-formed base pairs essential for Cas9 ribonucleoprotein (RNP) complex formation (Table 2).

sgRNA1 displayed a clearly defined stem-loop at the 3' end with a long unbranched stem (32 bp), two loops (one terminal hairpin and one internal bulge), minimal unpaired bases (3) in the guide region, and an MFE of −28.60 kcal/mol. **sgRNA2** showed a similar conformation with a 31 bp longest stem, two hairpin loops, 2 unpaired bases in the guide region, and an MFE of −27.40 kcal/mol. The zero self-complementarity scores for both guide sequences ensure minimal risk of misfolding that could impair Cas9 interaction. These structural properties are consistent with previously reported benchmarks for high-efficiency sgRNAs.

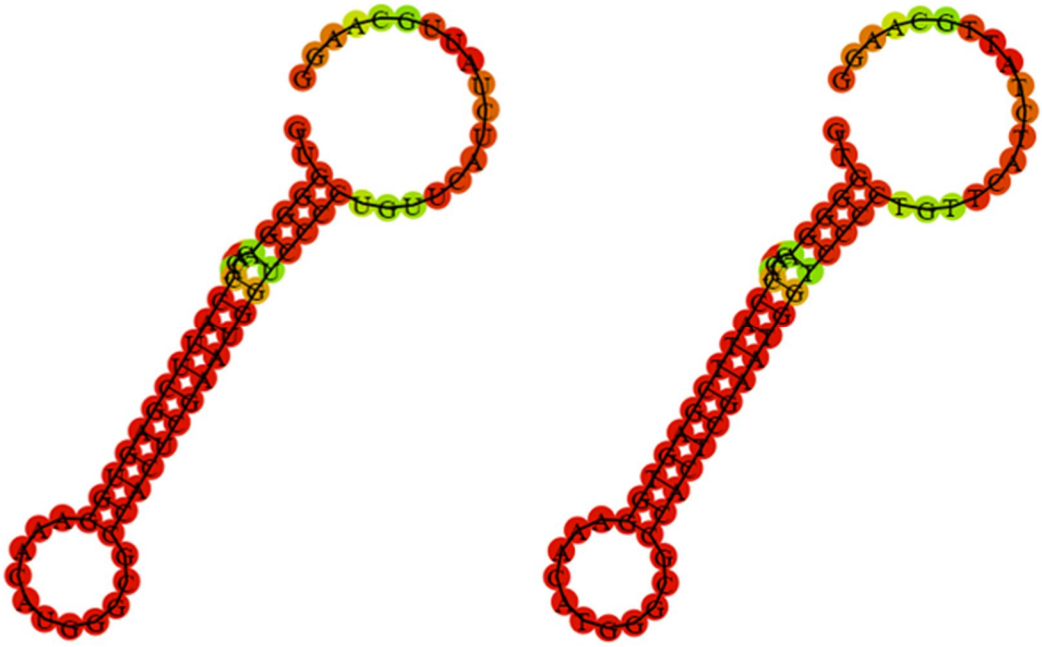


Figure 1: Predicted secondary structure of the predicted single-guide RNA- sgRNA1 & sgRNA2

Table 2. RNA secondary structure parameters of selected sgRNAs

Parameter	sgRNA 1	sgRNA 2
Total base pairs	55	53
Number of hairpin loops	2	2
Number of bulges/internal loops	1	1
Length of longest stem (bp)	32	31
Unpaired bases in guide region	3	2
Minimum free energy (kcal/mol)	−28.60	−27.40
Self-complementarity score	0	0
Predicted Cas9-binding compatibility	Compatible	Compatible

MFE: minimum free energy as calculated by RNAfold (ViennaRNA Package 2.0)

D. CRISPR/Cas9 Vector Construction

The in silico CRISPR/Cas9 vector was successfully designed using the pCas9-TPC backbone as a plant-compatible platform. Both sgRNAs were incorporated into distinct restriction sites (MluI/PacI for sgRNA1; SpeI/XbaI for sgRNA2) under the AtU6-26 promoter, which drives efficient sgRNA transcription in plant cells.

The vector included a donor DNA repair template with 500–800 bp homology arms flanking the target cleavage sites within VvMLO7, facilitating HDR-mediated precise gene insertion. A GFP reporter gene was incorporated at the SmaI site to enable visual screening of successful transformation events, simplifying downstream selection of transformed plant tissue.

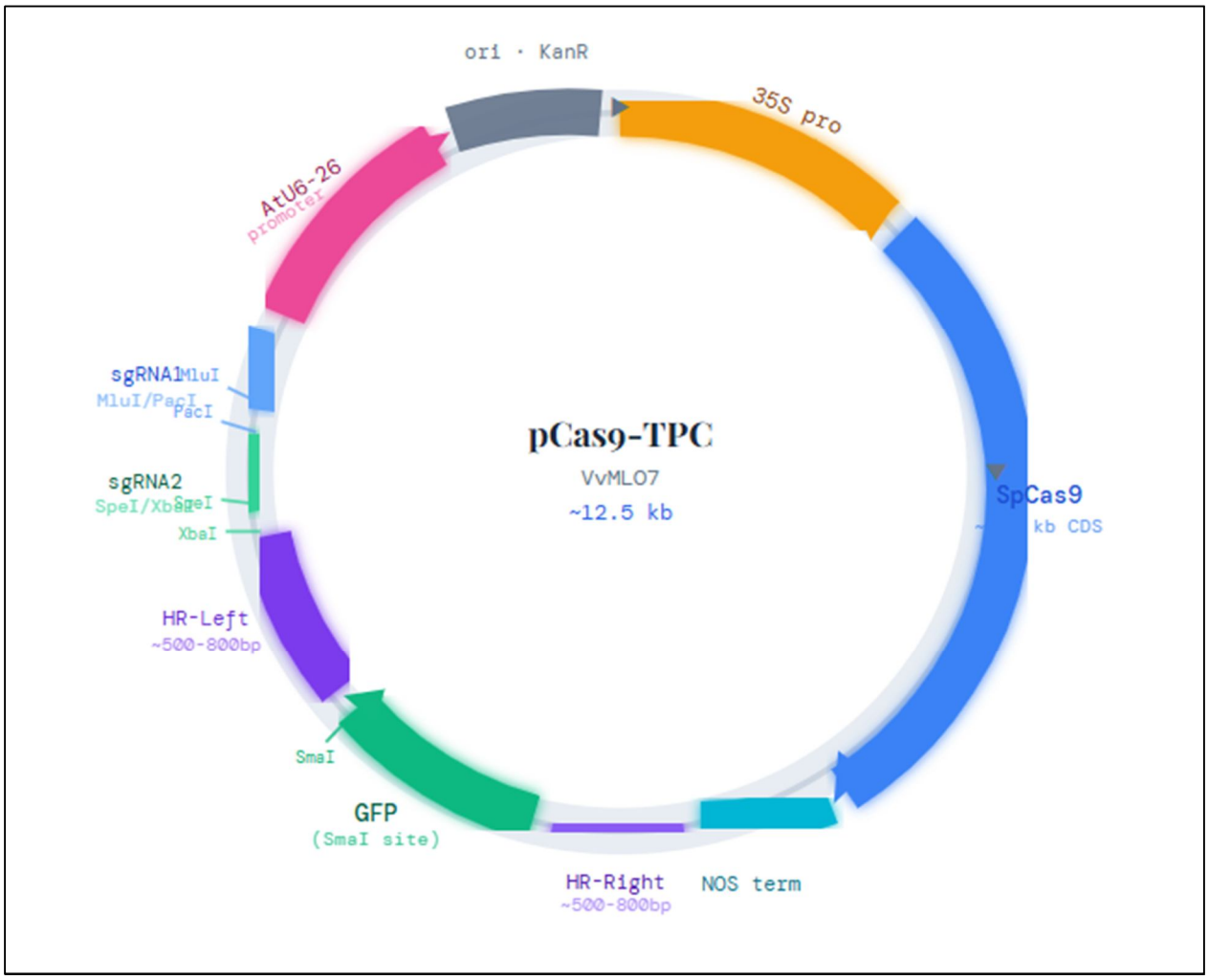


Figure 2: Circular plasmid map (~12.5 kb) with all features color-coded as arcs — CaMV 35S promoter, SpCas9 CDS, NOS terminator, AtU6-26 promoter, both sgRNA cassettes, homology arms (left & right), GFP reporter, and the backbone (ori + KanR), **Restriction sites labeled** — MluI, PacI (sgRNA1), SpeI, XbaI (sgRNA2), and SmaI (GFP), sgRNA sequences with GC content, efficiency scores, and strand direction

V. DISCUSSION

This study demonstrates a rigorous in silico pipeline for designing CRISPR/Cas9 components targeting VvMLO7 in grapevine, offering several advantages over conventional trial-and-error experimental approaches. The selection of VvMLO7 as the mutagenesis target is supported by its conserved transmembrane and calmodulin-binding domain architecture, consistent with MLO proteins characterized as S-genes in other plant species. The high conservation of MLO functional domains across plant lineages suggests that targeted disruption of VvMLO7 could recapitulate the durable, broad-spectrum resistance observed in other mlo mutants. The two selected sgRNAs exhibited favorable properties across multiple selection criteria. Their high on-target efficiency scores (65.95% and 59.05%), zero self-complementarity, and low off-target mismatch profiles make them strong candidates for experimental validation. Importantly, both target sequences within the VvMLO7 coding region are positioned to maximize the probability of frameshift mutations upon NHEJ repair, which would disrupt the protein reading frame and ablate MLO function. The thermodynamically stable secondary structures confirmed by RNAfold analysis indicate that both sgRNAs are capable of forming functional RNP complexes with Cas9.

The dual sgRNA strategy incorporated into the vector design provides an additional layer of flexibility. Operating independently, each sgRNA can introduce a frameshift-inducing indel at its respective target site. Operating synergistically, the two guides could excise an intervening genomic segment, further ensuring VvMLO7 knockout. The inclusion of a GFP selection marker significantly simplifies the identification of successful transformation events in grapevine tissue, which is otherwise challenging due to the species' recalcitrance to genetic manipulation.

The HDR-based knock-in strategy, while less efficient than NHEJ-mediated knockouts in plant systems, was selected to enable the precise introduction of defined sequences into the VvMLO7 locus. Previous research has demonstrated that HDR efficiency in plants can be enhanced through the use of geminivirus-based replicons, cell-cycle synchronization, and high-fidelity Cas9 variants [14]. Future experimental work should explore these strategies to optimize HDR rates in grapevine protoplasts or callus tissue.

A limitation of this study is that all analyses are computational. Experimental validation through protoplast transformation, PCR genotyping, Sanger sequencing, and phenotypic powdery mildew resistance assays will be essential to confirm the efficacy of the designed system. Potential off-target effects, while predicted to be minimal based on mismatch profiling, must also be experimentally assessed through whole-genome sequencing of edited lines. Additionally, grapevine's highly heterozygous genome and complex polyploidy may introduce challenges for uniform editing across all allelic copies.

VI. CONCLUSION

This study presents a comprehensive *in silico* framework for CRISPR/Cas9-mediated mutagenesis of the VvMLO7 susceptibility gene in *Vitis vinifera*. The pipeline encompasses target gene retrieval, sgRNA design and filtering, secondary structure validation, and expression vector construction. Two high-efficiency sgRNAs — GTGGGGACACACCATTCGAGTGG (65.95% efficiency) and TCCCGTGTTCACTTATTGCAAGG (59.05% efficiency) — were identified and confirmed to form stable secondary structures compatible with Cas9 binding. A complete expression vector incorporating dual sgRNAs, an HDR donor template, and a GFP selection marker was designed in silico using the pCas9-TPC backbone.

This work provides a rational, cost-effective foundation for experimental validation and represents a significant step toward developing powdery mildew-resistant grapevine cultivars through precision genome editing. The elimination or reduction of MLO7 susceptibility gene function may confer durable, chemical-free resistance, reducing the ecological footprint of grapevine cultivation and advancing sustainable viticulture practices. Future efforts should focus on experimental transformation, phenotypic validation of resistance, and assessment of potential off-target effects in edited plant lines.

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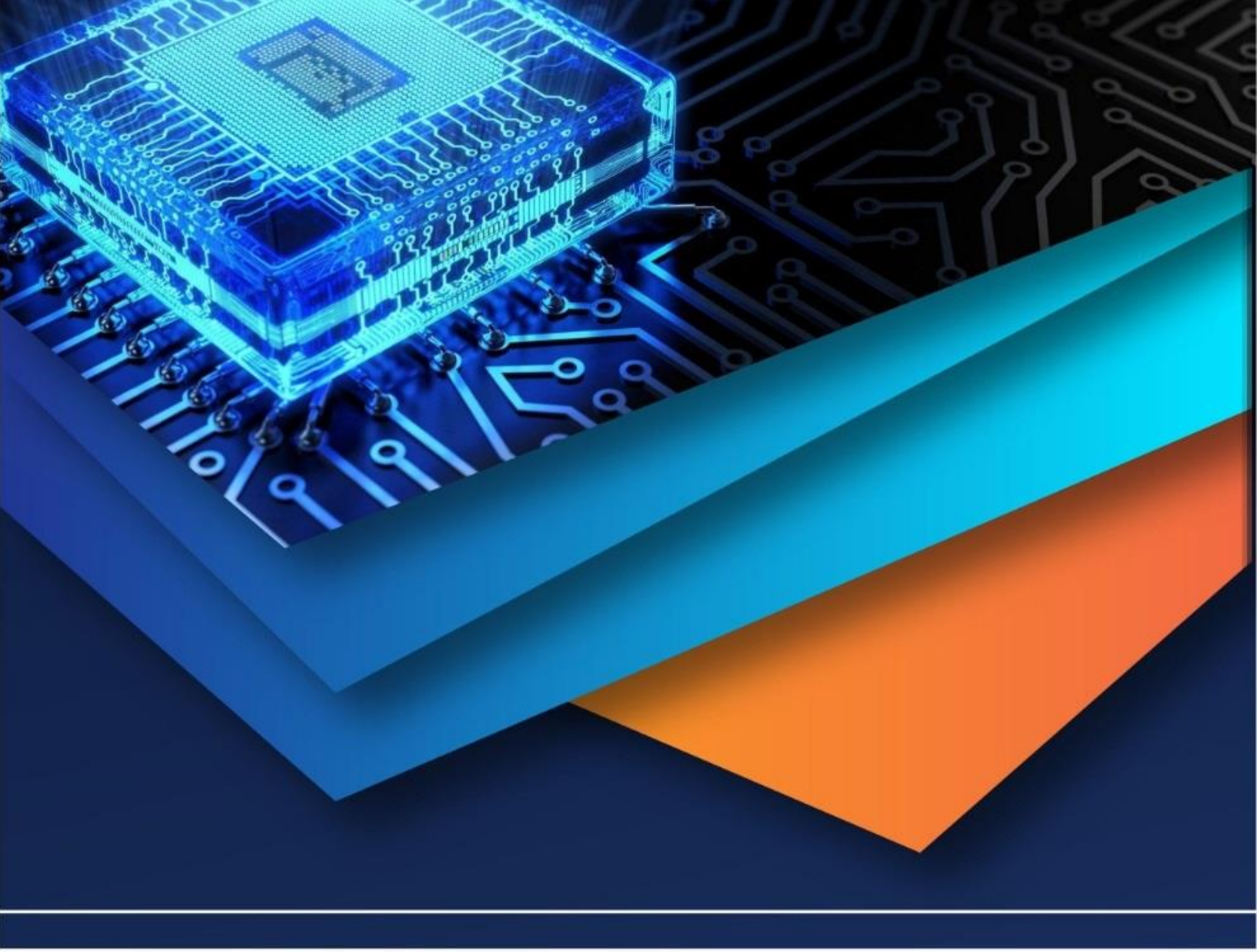
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