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IN SILICO DESIGN AND TARGET VERIFICATION OF CRISPR-CAS9 gRNAs FOR TaPPRT1 KNOCKOUT IN UZBEK BREAD WHEAT (*TRITICUM AESTIVUM* L.)

Makhmudova Maftuna Uktam kizi, Turayev Ozod Sunnatliyevich

Research Institute of Plant Genetic Resources

Korabekova Shakhnoza Muxiddinovna

Tashkent Institute of Chemical Technology

Annotatsiya.

Ubiquitin-26S proteasoma tizimi (UPS) ekinlarda abiotik stress omillariga bo'lgan javob reaksiyalarini tartibga soluvchi muhim yo'l bo'lib xizmat qiladi, bunda E3 ubiquitin ligaza PPRT1 geni absorsion kislota (ABA) signallari uzatilishi hamda osmotik chidamlilikning asosiy negativ regulatori (tormozlovchisi) hisoblanadi. CRISPR-Cas9 genom tahrirlash texnologiyasi yordamida ushbu regulatoryni nokaut qilish istiqbolli yumshoq bug'doy (*Triticum aestivum* L.) navlarida qurg'oqchilikka chidamlilikni oshirishning aniq yo'lini taklif etadi. Biroq, bug'doy genomining murakkab geksaploid tabiati va mahalliy navlardagi mavjud bir nukleotidli polimorfizmlar (SNP) maqsadli genni o'chirish samaradorligini pasaytirishi mumkin. Ushbu tadqiqot barcha uchta (A, B va D) subgenom bo'ylab TaPPRT1 genining 2-ekzonini destruksiya qilishga qaratilgan yagona yo'naltiruvchi RNK (sgRNK) molekularini strategik in silico loyihalash, tarkibiy baholash va eksperimental maqsadli sayt (target-site) korreksiyasiga qaratilgan. CRISPR dasturi yordamida uchta gRNK loyihalashtirildi va ularning ichki halqasimon (hairpin) tuzilmalarini hosil qilmasligi RNAfold dasturi orqali tahlil qilindi. Mahalliy navlarning Sanger sekvenirlash natijalari shuni ko'rsatdiki, gRNA3 ning maqsadli lokusida Chinese Spring referens genomiga nisbatan genomik nomuvofiqlik mavjud bo'lib, bu barcha gomeologik nusxalarning to'liq nokaut qilinishini ta'minlash uchun dizaynga maqsadli o'zgartirish kiritishni talab qildi. Bizning natijalarimiz keng ko'lamli genetik transformatsiyalarni boshlashdan oldin, dastlabki nazorat nuqtasi sifatida navga xos ketma-ketlikni (ketma-ketlik verifikatsiyasini) eksperimental tekshirish mutloq zarurligini ko'rsatadi.

Kalit so'zlar:

Triticum aestivum, CRISPR-Cas9, TaPPRT1, gRNK dizayni, polimorfizm, qurg'oqchilikka chidamlilik.

Аннотация.

Убиквитин-26S протеасомная система (UPS) служит важным путем, регулирующим ответы культур на абиотический стресс, при этом E3 убиквитинлигаза PPRT1 выступает в качестве ключевого негативного регулятора сигналинга абсцизовой кислоты (АБК) и осмотической толерантности. Деактивация этого регулятора с помощью редактирования генома CRISPR-Cas9 предлагает точный путь повышения устойчивости к засухе у элитных сортов мягкой пшеницы (*Triticum aestivum* L.). Однако сложная гексаплоидная природа пшеницы и лежащие в ее основе однонуклеотидные полиморфизмы (SNP) у местных сортов могут снизить эффективность расщепления мишени. Данное исследование сосредоточено на стратегическом in silico дизайне, структурной оценке и экспериментальной верификации сайт-мишеней единичных направляющих РНК (sgRNK), предназначенных для деградации экзона 2 гена TaPPRT1 во всех трех (A, B и D) субгеномах. Три гRNK были сконструированы с использованием CRISPR и оценены с помощью RNAfold для предотвращения образования внутримолекулярных шпилек. Сэнгеровское секвенирование местных узбекских сортов выявило критическое геномное несоответствие в локусе мишени gRNA3 по сравнению с референсным геномом Chinese Spring, что потребовало направленной модификации дизайна для обеспечения полного нокаута всех гомеологов. Наши результаты подчеркивают абсолютную необходимость сортоспецифичной верификации последовательностей в качестве предварительного контрольного этапа перед началом крупномасштабных генетических трансформаций.

Ключевые слова:

Triticum aestivum, CRISPR-Cas9, TaPPRT1, дизайн гRNK, полиморфизм, засухоустойчивость.

Abstract.

The Ubiquitin-26S Proteasome System (UPS) serves as a vital pathway regulating abiotic stress responses in crops, with the E3 ubiquitin ligase PPRT1 acting as a key negative regulator of abscisic acid (ABA) signaling and osmotic tolerance. Deactivating this regulator via CRISPR-Cas9 genome editing offers a precise path to enhancing drought resilience in elite bread wheat (*Triticum aestivum* L.). However, the complex hexaploid nature of wheat and underlying single nucleotide polymorphisms (SNPs) in local varieties can compromise target cleavage efficiency.

This study focuses on the strategic in silico design, structural evaluation, and experimental target-site verification of single-guide RNAs (sgRNAs) tailored to disrupt Exon2 of the TaPPRT1 gene across all three (A, B, and D) subgenomes. Three gRNAs were designed using CRISPR and evaluated via RNAfold to prevent structural hairpins. The Sanger sequencing of local Uzbek cultivars revealed a critical genomic mismatch in the target locus of gRNA3 compared to the Chinese Spring reference genome, requiring a targeted design modification to ensure complete homoeolog disruption. Our results highlight the absolute necessity of variety-specific sequence verification as a preliminary checkpoint before embarking on large-scale genetic transformations.

Keywords:

adaptive learning, artificial intelligence, gamification, machine learning, Bayesian network, motivation, educational algorithm, intelligent system, personalized education, learning environment.

1. Introduction. Bread wheat (*Triticum aestivum* L.) is a foundational global staple crop, yet its production is increasingly vulnerable to severe, accelerating drought cycles. These

environmental stressors severely limit grain yield, protein deposition, and gluten quality, posing a critical threat to food security in arid and semi-arid regions such as Uzbekistan. Traditional breeding methodologies struggle to keep pace with these changing climates due to the genetic complexity of wheat's allohexaploid genome ($2n=6x=42$, AABBDD). Consequently, functional genomics and precision genome editing have emerged as essential strategies to accelerate the development of climate-resilient elite germplasm [3; 6].

Confronted with osmotic and oxidative stress, plants rely heavily on intracellular signaling cascades to modulate their physiological defenses. Recent research points toward the Ubiquitin-26S Proteasome System (UPS) as a central hub for editing stress-responsive protein accumulation [5; 9]. Specifically, the Ring-type E3 ubiquitin ligase, *PPRT1* (Poly(A)-specific ribonuclease-like protein), has been identified as a significant negative regulator of drought tolerance. *PPRT1* down-regulates or moderates the plant's sensitivity to endogenous Abscisic Acid (ABA), the primary hormone governing stomatal closure, root architecture adjustments, and protective osmolyte accumulation [7]. By knocking out or disrupting the functional domains of the *TaPPRT1* gene, the molecular brakes on the ABA survival pathway can be lifted, theoretically allowing the plant to sustain metabolic homeostasis under severe moisture deficits.

The invention of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 (Cas9) technology provides a highly efficient method for generating such targeted knockouts [1]. To achieve a complete phenotype change in hexaploid wheat, all six copies (homoeologs) of the target gene across the A, B, and D subgenomes must typically be disrupted. This requires the design of multiplex single-guide RNAs (sgRNAs) that target highly conserved exon sequences [6; 8].

However, a major bottleneck in moving from *in silico* computer models to successful live plant transformations is the genetic divergence between international reference genomes and local, adapted cultivars. Most online gRNA design tools rely almost exclusively on the reference genome of the model variety *Chinese Spring* [4]. Local elite varieties adapted to unique regional soils often carry unmapped Single Nucleotide Polymorphisms (SNPs). If an unmapped SNP falls within the guide RNA's target spacer or its adjacent Protospacer Adjacent Motif (PAM), Cas9 binding can fail completely, resulting in incomplete subgenome knockouts [2].

To address this challenge, this study establishes a rigorous pre-validation pipeline. We describe the *in silico* selection and thermodynamic profiling of three gRNAs targeting Exon 2 of *TaPPRT1*, paired with targeted sequencing to verify and correct these target loci within our local lines.

2. Materials and Methods

2.1. CRISPR-Cas9 gRNA Design and In Silico Validation

To achieve a targeted knockout of the *PPRT1* gene, three single-guide RNAs (gRNAs) were designed specifically targeting Exon2 using the CRISPOR tool (<https://crispor.gi.ucsc.edu/>). Given the hexaploid nature of bread wheat, gRNAs were strategically selected to ensure coverage across all three subgenomes:

gRNA1: Designed for specificity to the A subgenome.

gRNA2&3: Designed to target conserved regions in the B and D subgenomes.

The gRNAs were rigorously screened for GC content (aiming for 40–60%) and potential off-target mismatches within the *Triticum aestivum* genome. Furthermore, the RNAfold web server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) was utilized to

predict the secondary structure and minimum free energy of the gRNA-scaffold complex, ensuring the spacers do not form internal hairpins that could inhibit Cas9 binding.

2.2. Target Site Verification via Sanger Sequencing

To ensure the gRNAs perfectly match the sequences of local Uzbek wheat varieties, Sanger sequencing was performed on the amplicons flanking Exon 2.

2.2.1. PCR Product Enzymatic Cleanup

Following target locus amplification via Polymerase Chain Reaction (PCR), enzymatic purification of the post-PCR products was performed to remove remaining single-stranded oligonucleotide primers and unincorporated dNTPs prior to downstream sequence analysis. Cleanup was executed using ExoSAP-IT™ PCR Product Cleanup reagent (Applied Biosystems, Thermo Fisher Scientific, USA). For each reaction, 5 µl of post-PCR mixture was combined directly with 2 µl of the ExoSAP-IT™ reagent on ice, establishing a final reaction volume of 7 µl. The enzymatic reaction was carried out in a thermal cycler using a two-step incubation profile: first, the mixture was incubated at 37°C for 15 minutes to allow hydrolytic degradation of excess primers and nucleotides, followed immediately by incubation at 80°C for 15 minutes to thoroughly inactivate the enzymes.

2.2.2. Cycle Sequencing Reaction

Cycle sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The reactions were conducted in a total volume of 10 µl per sample. Each reaction mixture was prepared on ice and contained 2.0 µl of the Ready Reaction Premix (2.5X), 1.0 µl of BigDye™ Sequencing Buffer (5X) to maintain a final working concentration of 1X, 3.2 pmol of target-specific forward or reverse sequencing primers, and the purified DNA template (diluted proportionally according to fragment size), with the total volume adjusted to 10 µl using sterile deionized water.

The reaction mixtures were thoroughly homogenized, briefly centrifuged, and subjected to automated thermal cycling in a calibrated thermal cycler. The cycle sequencing program initiated with a preliminary denaturation step at 96°C for 1 minute, followed by 25 consecutive cycles of rapid thermal denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. A strict thermal ramping profile of 1°C per second was maintained across all stages to eliminate background noise.

2.2.3. Sequencing Reaction Purification

Post-cycle sequencing extension products were purified using the BigDye XTerminator™ Purification Kit (Applied Biosystems, USA) to effectively sequester unincorporated dye terminators, dNTPs, and inorganic salts prior to capillary electrophoresis. Based on the 10 µl initial cycle sequencing volume, the purification reagents were added proportionally in a 96-well reaction plate. Prior to pipetting, the BigDye XTerminator™ Matrix was thoroughly vortexed to ensure a completely homogeneous suspension.

To each 10 µl reaction, 45 µl of SAM™ Solution and 10 µl of the resuspended BigDye XTerminator™ Matrix were directly dispensed. The plate was subsequently sealed tightly and subjected to high-speed vortexing on a specialized digital vortex mixer at 2000 rpm for 20 minutes to achieve complete capture of impurities. Following vortexing, the plate was briefly centrifuged at 1000 xg for 2 minutes at room temperature to pellet the matrix-bound contaminants. The supernatant containing the purified, dye-terminated DNA fragments was directly loaded into the Genetic Analyzer system for capillary electrophoresis.

2.2.4. Sequence Alignment and Target Site Analysis

The resulting chromatograms were analyzed, and the sequences from the 9 local varieties were aligned against the Chinese Spring (IWGSC RefSeq v2.1) reference sequences for the A, B, and D subgenomes using SnapGene (Figure 1).



Figure 1. The alignment of *PPRT1* Exon2 of local varieties to that of Chinese spring.

The alignment was used to confirm the presence of the PAM sequence (NGG) and to identify any Single Nucleotide Polymorphisms (SNPs) in the local varieties that might interfere with gRNA hybridization.

3. Results. While gRNA1 (A-genome specific) and gRNA2 (B&D-genome targeted) showed 100% homology with the Sanger sequences of our local varieties, the initial design for gRNA3 revealed a mismatch in the target region of the local genotypes compared to the Chinese Spring reference. To maintain high cleavage efficiency, gRNA3 was redesigned to perfectly match the local allele identified in our sequencing data. All guides target Exon2 (Table 1).

Table 1. Designed gRNAs with overhangs added

gRNAs	Sequence 5`-3`	Target
TaPPRT1-gRNA1F	TGGCACTCCATGAAATAGCCATAG	Exon2
TaPPRT1-gRNA1R	AAACCTATGGCTATTTTCATGGAGT	
TaPPRT1-gRNA2F	TGGCACTCCATGAAATAGCCACAG	
TaPPRT1-gRNA2R	AAACCTGTGGCTATTTTCATGGAGT	
TaPPRT1-gRNA3F	CTTGGCAGCCCTTCATTACCTCA	
TaPPRT1-gRNA3R	AAACTGAGGTAATGAAGGGGCTGC	

4. Discussion. The decision to redesign gRNA3 based on local sequencing data is essential for the transition from in silico design to in vivo application. In wheat, subgenome-specific mutations are often the goal to study dosage effects, but for a complete knockout of a negative regulator like *PPRT1*, all functional homoeologs must be disrupted. The presence of SNPs in local varieties is a common hurdle in translating CRISPR technology from model genotypes like Chinese Spring or Fielder to locally adapted elite lines. By correcting gRNA3, we avoid escapes where one subgenome remains functional, which would likely mask the drought-tolerant phenotype we aim to achieve.

5. Conclusion. This study successfully established a reliable, variety-specific pre-validation pipeline for the target optimization of CRISPR-Cas9 components against the abiotic

stress gene *TaPPRT1* in bread wheat. Through computational modeling via CRISPOR and RNAfold, three highly specific gRNAs targeting Exon2 were characterized to support clean subgenome coverage while avoiding deleterious secondary hairpin structures.

Our subsequent experimental sequence verification proved to be a critical step: while gRNA1 and gRNA2 maintained perfect homology with our local elite Uzbek cultivars, the original reference-designed gRNA3 displayed a definitive sequence mismatch within the local germplasm's genomic target zone. By successfully re-engineering gRNA3 to create a perfect match with the local allele, we bypassed the risk of incomplete homoeologic cleavage and off-target escape.

Ultimately, these findings demonstrate that relying solely on model reference sequences like *Chinese Spring* can decrease multiplex editing efficiency in regional varieties. The fully optimized and verified gRNA library developed in this work establishes a solid foundation for upcoming transformation experiments aimed at improving drought tolerance in local wheat varieties.

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ANIQ VA TABIIY FANLAR

Zaparov A.A. <i>Nazariy mexanika fanini o'qitishda multimediali o'qitish texnologiyasidan foydalanish</i>	141	145
Ergashev Biloldin Mirsharipjon o'g'li, Yuldashev Otabek Ravshan o'g'li <i>Talabalarning mustaqil ta'lim olish ko'nikmalarini rivojlantirish: tahlil va samarali strategiyalar</i>	146	155
Soliyev Iqboljon Maxammadjonovich, Boboyev Akramjon Yo'ldashboyevich <i>GaAs tagliklarda suyuq fazadan epitaksiya usuli bilan o'stirilgan (GaAs)_{1-x}-y(Ge₂)_x(ZnSe)_y qattiq qorishmali yupqa qatlamlarning strukturaviy va fazaviy xususiyatlarini tadqiq etish</i>	156	163
Махсудов Одилжон Хусанович <i>Экономический анализ малых предприятий в сфере услуг</i>	164	168
Jaloldinova Sh.X., Kambarova M.M. <i>Materialshunos fanini o'qitishning zamonaviy usullari</i>	169	170
Zulunova Moxlaroyim Abdurashid qizi <i>Atom va yadro fizikasi tushunchalarini tushuntirishda multimedia vositalari asosida zamonaviy dars dizaynini takomillashtirish</i>	171	174
Jaloldinova Shaxnozaxon Xusanboyevna, Mir-yusupova Muhayyoxon Alimjanovna <i>Texnologiya fani darolarida xalq hunarmandchiligi bo'limini o'qitishda innovatsion yondashuvlar</i>	175	177
Komilov Murodjon Muxtarjon o'g'li, Mirzaalimov Avazbek Alisherovich, Mirzaalimov Navro'zbek Alisherovich, Rashidov Bobur Dilmurodovich, Mirzaalimova Mavluda Sahibovna <i>Laboratoriya mashg'ulotlarida qo'llaniladigan bir yarim davrli to'g'irilagichdan o'tayotgan kuchlanishni aniqlash</i>	178	181
Jo'rayev Farxodbek Murodjon o'g'li <i>Sun'iy intellekt va gamifikatsiyani birlashtirgan adaptiv o'qitish muhitini loyihalash: arxitektura va algoritmlar</i>	182	187
Makhmudova Maftuna Uktam kizi, Korabekova Shakhnoza Muxiddinovna, Turayev Ozod Sunnataliyevich <i>In silico design and target verification of crispr-cas9 grnas for tapprt1 knockout in uzbek bread wheat (Triticum aestivum L.)</i>	188	192
Arslanov D.M., Xalikov Q.K., Gapparov B.M. <i>O'simliklarning abiotik stress omillariga chidamliligini oshirishning molekulyar mexanizmlari va biotexnologik yondashuvlari</i>	193	203
Atajonov Muxiddin Odiljonovich <i>Feototermobatareya olish usulini simulink dasturida Modellashtirish metodi</i>	204	208
Xolmatova X., Aliyev R., Mirzaalimov A., Rashidov B., Mirzaalimov N., Odiljonov A. <i>Mobil quyosh energetik tizimida konsentratsion reflektor va turli fonlarning fotoelektrik samaradorlikka ta'sirini aniqlash va tadqiq etish</i>	209	214

