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REGULATORY ELEMENTS GOVERNING CRISPR/CAS9 EFFICACY IN PLANT BIOTECHNOLOGY

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Annotation. *The CRISPR/Cas9 system has revolutionized plant genome engineering by enabling precise, efficient, and programmable genetic modification. However, genome-editing efficiency is strongly influenced by vector design, particularly by regulatory elements such as promoters and terminators. These elements govern transcription, RNA stability, and protein expression. This article provides a comprehensive analysis of the promoter and terminator systems in plant CRISPR/Cas9 constructs, focusing on monocot–dicot differences, transcriptional regulation, and vector optimization strategies. In this review, we discuss recent advances in synthetic promoter engineering and transcriptional control. Understanding these regulatory components is essential for maximizing editing efficiency and expanding CRISPR applications in plant biotechnology.*

Key words: *CRISPR/Cas9, plant genome editing, promoter architecture, terminator sequences, vector optimization, transcriptional regulation, monocot-dicot differences, synthetic biology*

РЕГУЛЯТОРНЫЕ ЭЛЕМЕНТЫ, ОПРЕДЕЛЯЮЩИЕ ЭФФЕКТИВНОСТЬ CRISPR/CAS9 В БИОТЕХНОЛОГИИ РАСТЕНИЙ

Аннотация. *Система CRISPR/Cas9 произвела революцию в инженерии генома растений, обеспечив точные, эффективные и программируемые генетические модификации. Тем не менее, эффективность редактирования генома во многом определяется конструкцией вектора, в частности регуляторными элементами — промоторами и терминаторами. Эти элементы управляют транскрипцией, стабильностью РНК и уровнем экспрессии белков. В данной статье представлен комплексный анализ промоторных и терминаторных систем в конструкциях CRISPR/Cas9 для растений с акцентом на различия между однодольными и двудольными растениями, транскрипционную регуляцию и стратегии оптимизации векторов. Также рассматриваются последние достижения в области инженерии синтетических промоторов и транскрипционного контроля. Понимание этих регуляторных компонентов необходимо для максимизации эффективности редактирования и расширения возможностей применения CRISPR в биотехнологии растений.*

Ключевые слова: *CRISPR/Cas9, редактирование генома растений, архитектура промотора, последовательности терминаторов, оптимизация вектора, транскрипционная регуляция, различия между однодольными и двудольными, синтетическая биология*

O‘ SIMLIKLAR BIOTEXNOLOGIYASIDA CRISPR/CAS9 SAMARADORLIGINI BOSHQARUVCHI REGULYATOR ELEMENTLAR

Annotatsiya. *CRISPR/Cas9 tizimi aniq, samarali va dasturlashtiriladigan genetik o‘ zgartirishlarni amalga oshirish imkonini berish orqali o‘ simlik genom muhandisligi sohasida inqilob yasadi. Biroq, genom tahrirlash samaradorligi vektor dizayni, xususan promotorlar va terminatorlar kabi regulyator elementlar bilan bevosita bog‘ liq. Ushbu elementlar transkripsiya, RNK barqarorligi va oqsil ekspressiyasi darajalarini boshqaradi. Ushbu maqolada o‘ simliklar uchun CRISPR/Cas9 konstruksiyalarida promotor va terminator tizimlarining keng qamrovli tahlili keltirilgan bo‘ lib, bir urug‘ pallali va ikki urug‘ pallali o‘ simliklar o‘ rtasidagi farqlar, transkripsion regulyatsiya va vektor optimallashtirish strategiyalariga e’tibor qaratilgan. Sintetik promotor muhandisligi va transkripsion nazorat sohasidagi so‘ nggi yutuqlar ham muhokama qilinadi. Ushbu regulyator komponentlarni tushunish tahrirlash samaradorligini oshirish va o‘ simlik biotexnologiyasida CRISPR imkoniyatlarini kengaytirish uchun muhim ahamiyat kasb etadi.*

Kalit so‘ zlar: *CRISPR/Cas9, o‘ simlik genomini tahrirlash, promotor dizayni, terminator ketma-ketliklari, vektor optimallashtirilishi, transkripsion regulyatsiya, bir urug‘ pallali va ikki urug‘ pallali o‘ simliklar o‘ rtasidagi farqlar, sintetik biologiya*

Introduction.

The advent of the CRISPR/Cas9 system has revolutionized genome engineering by offering unprecedented simplicity, high efficiency, and broad applicability across diverse biological systems, including plants. As a transformative tool in agricultural biotechnology, CRISPR/Cas9 holds immense potential to address global food security challenges by enabling the development of crop varieties with enhanced yields, superior quality, and robust resistance to biotic and abiotic stress [23, 24]. Mechanistically, the system relies on a Cas9 nuclease and guide RNA (gRNA), which assemble into a ribonucleoprotein (RNP) complex capable of inducing precise double-strand breaks (DSBs) at predefined genomic loci [6]. Following DSB formation, endogenous DNA repair pathways, predominantly non-homologous end joining (NHEJ) and homologous recombination (HR), are harnessed to introduce targeted genetic modifications into the genome.

While the meticulous design of single guide RNA (sgRNA) is paramount for ensuring target site specificity, emerging empirical evidence highlights the profound impact of overall vector architecture on genome-editing efficacy [1]. Specifically, the selection of regulatory elements, such as promoters and terminators, is critical for optimizing the expression of CRISPR machinery in vivo. In plant systems, the pronounced divergence in transcriptional machinery between monocotyledonous and dicotyledonous species necessitates the tailored selection and refinement of these regulatory elements. For instance, recent optimization studies have demonstrated that replacing ubiquitous promoters with species-specific endogenous ones, such as substituting the CaMV-35S promoter with the UBQ10 promoter in specific dicots or using endogenous U3/U6 promoters in target crops such as grape, can yield multiple-fold enhancements in targeted mutation frequencies [25,26]. Consequently, the strategic configuration and synergistic combination of optimal promoters and terminators are pivotal for maximizing CRISPR/Cas9-mediated editing outcomes across diverse plant platforms [2].

Promoter Systems in CRISPR/Cas9 Vectors. Promoters are regulatory DNA sequences located upstream of genes that control transcription initiation by recruiting RNA polymerases and transcription factors. They determine the timing, location, and intensity of gene expression [4]. In plant CRISPR/Cas9 vectors, promoters regulate two essential components: Cas9 nuclease expression and sgRNA transcription. The effectiveness of genome editing is therefore highly dependent on promoter strength and compatibility with host transcriptional machinery. Cas9 is a protein-coding gene transcribed by RNA polymerase II (Pol II), producing mRNA that is translated into functional Cas9 protein.

In dicot plants, the Cauliflower Mosaic Virus 35S (CaMV35S) promoter is widely used due to its strong constitutive activity (Odell et al., 1985). However, in monocots, promoters such as maize ubiquitin (ZmUbi) and rice actin are preferred because they exhibit higher transcriptional efficiency in cereal crops [3]. The selection of an appropriate promoter directly influences Cas9 expression levels and genome editing outcomes. Strong constitutive promoters typically result in higher mutation frequencies due to sustained Cas9 activity [16]. sgRNAs are short non-coding RNAs (~100–200 nucleotides) and are transcribed by RNA polymerase III (Pol III). These promoters are specialized for small RNA transcription and ensure precise transcript boundaries. Common sgRNA promoters include: arabidopsis U6/U3 promoters (dicots), rice U6/U3 promoters (monocots). Pol III promoters are essential for producing functional sgRNAs with correct 5′ and 3′ ends, which are critical for Cas9 targeting efficiency [21].

Promoters in CRISPR systems can be categorized based on expression patterns: **Constitutive promoters** drive continuous gene expression across tissues and developmental stages. They are widely used for high-efficiency genome editing [22]. **Tissue-specific promoters** enable targeted gene editing in specific organs, reducing unintended effects in

non-target tissues. **Inducible promoters** allow temporal control of gene expression in response to external stimuli such as chemicals or environmental conditions. **Synthetic promoters** engineered promoters designed to enhance expression strength, support multiplex sgRNA expression, and improve regulatory precision. These are increasingly used in advanced CRISPR applications [7].

Terminator Systems in CRISPR/Cas9 Vectors. Terminators are DNA sequences located downstream of genes that signal the end of transcription. They play a critical role in: mRNA cleavage and polyadenylation, transcript stability, prevention of transcriptional read-through. The combination of promoter and terminator sequences strongly influences gene expression levels [2]. Cas9 expression requires Pol II terminators to ensure proper mRNA processing and stability. Common terminators include: **NOS terminator** (widely used, stable across species), **OCS terminator** (reduces transcriptional read-through) **35S terminator** (often paired with CaMV35S promoter). Efficient terminators enhance mRNA stability, increase translation efficiency, and improve overall genome editing performance [8]. Pol III transcription terminates at short thymidine (poly-T) sequences, typically consisting of five consecutive thymidines (TTTTT). This mechanism ensures precise sgRNA termination and functionality. Typical sgRNA cassette: U6 promoter →sgRNA →poly-T terminator. This simple and efficient termination system is critical for accurate CRISPR activity [21].

Terminators influence genome editing efficiency through several mechanisms: **mRNA stability:** Strong terminators increase transcript half-life and protein production. **Polyadenylation efficiency:** Essential for nuclear export and translation. **Transcriptional isolation:** Prevents interference between adjacent expression cassettes. **Gene silencing reduction:** Particularly important in monocot plants. Optimized terminator selection can significantly enhance CRISPR efficiency [9].

Monocot vs Dicot Differences in CRISPR Vector Design. Promoter efficiency is highly species-dependent due to differences in transcriptional machinery. Dicots commonly use CaMV35S promoters, whereas monocots rely on ubiquitin-based promoters for optimal expression [3]. Although terminators are generally less species-specific, endogenous terminators may improve expression efficiency and reduce gene silencing in monocot systems [9]. CRISPR/Cas9 plasmid vectors are not limited to genome editing components (Cas9 and sgRNA) but also include a variety of auxiliary genetic elements that are essential for plasmid propagation, selection, expression, and intracellular targeting. These backbone components play a critical role in ensuring the stability, functionality, and efficiency of CRISPR systems in both bacterial and plant cells [12,16].

Before plant transformation, CRISPR vectors are typically constructed and amplified in bacterial systems, such as Escherichia coli. Therefore, plasmids must contain elements that allow their replication and selection in bacteria. Antibiotic resistance genes, such as the ampicillin resistance gene (AmpR), encode β-lactamase, an enzyme that degrades ampicillin and allows only transformed bacterial cells to survive in selective media. This selection system is essential for isolating plasmids carrying CRISPR constructs during cloning [17]. **Origin of Replication (Ori)** the origin of replication is a DNA sequence that enables autonomous plasmid replication within bacterial cells. Common origins such as ColE1 determine plasmid copy number, which influences DNA yield and cloning efficiency. High-copy origins produce large amounts of plasmid DNA, whereas low-copy origins improve stability for large CRISPR constructs [11]. **Rop (Repressor of Primer Protein)** the Rop protein regulates plasmid copy number by stabilizing RNA–RNA interactions involved in replication initiation. This regulation is particularly important in large CRISPR vectors, as excessive copy numbers may lead to recombination or instability [15].

Some CRISPR vectors include bacterial regulatory systems that allow the controlled expression of Cas9 or other proteins during cloning or protein production. Lac Operon Components (Lac Promoter, Lac Operator, LacI) The lac operon system provides inducible control of gene expression in bacterial cells. The lac promoter (Plac) drives transcription, whereas the lac operator (LacO) serves as a binding site for the LacI repressor protein. In the absence of an inducer, LacI binds to LacO and prevents transcription. Upon the addition of IPTG or lactose, LacI dissociates, allowing gene expression [13]. This system is useful for: Preventing toxic overexpression of Cas9 in bacteria and controlling recombinant protein production. **T7 Promoter System** the T7 promoter is a strong bacteriophage-derived promoter recognized by T7 RNA polymerase. It enables high-level transcription and is commonly used in *E. coli* strains such as BL21(DE3) for protein production [19]. Although not typically used for plant expression, the T7 system is valuable for: In vitro sgRNA synthesis, recombinant Cas9 protein purification. Efficient protein production requires proper translation initiation signals. **Ribosome Binding Site (RBS)** the RBS, also known as the Shine–Dalgarno sequence, is essential for initiating translation in prokaryotes. It aligns the ribosome with the start codon, ensuring proper translation of Cas9 or other proteins in bacterial systems [18]. Without an RBS, even correctly transcribed mRNA cannot be translated into protein, highlighting its importance in vector design. For CRISPR/Cas9 to function in plant cells, the Cas9 protein must be localized to the nucleus, where genomic DNA resides. **Nuclear Localization Signals (NLS)** nuclear localization signals, such as the SV40 NLS, are short peptide sequences rich in lysine and arginine residues that direct proteins to the nucleus via the nuclear import machinery. Cas9 is often engineered with one or two NLS sequences (NLS–Cas9–NLS) to ensure

efficient nuclear targeting [14]. Efficient nuclear localization is critical because: Genome editing occurs in the nucleus and reduced nuclear import decreases editing efficiency.

Certain CRISPR constructs include protein tags for biochemical applications. **6×His Tag (Hexahistidine Tag)** the 6×His tag is a short peptide sequence that binds to nickel or cobalt ions, allowing affinity purification of recombinant proteins. This tag is widely used for: Cas9 protein purification, western blot detection and structural and biochemical studies [10]. Although not required for genome editing in vivo, tagging is essential in experimental workflows involving protein characterization. The overall performance of CRISPR/Cas9 systems depends on the coordinated interaction between backbone elements and expression cassettes. While promoters and terminators control transcription, backbone components ensure plasmid stability, efficient cloning, and proper protein function. For example: A stable origin of replication ensures sufficient plasmid yield, antibiotic selection guarantees successful cloning, NLS sequences ensure Cas9 reaches the nucleus, regulatory systems prevent toxic expression in bacteria. Thus, backbone components indirectly but critically influence genome editing efficiency [12]. While designing CRISPR vectors, the following factors should be considered: plasmid size (larger constructs reduce transformation efficiency), copy number (must balance yield and stability), compatibility with the host system (bacterial vs. plant expression requirements), and recombination risk (minimized by controlled copy number and stable elements). Optimizing these parameters improves the cloning efficiency, vector stability, and ultimately, the genome editing performance. Successful CRISPR/Cas9 applications require careful optimization of vector components, including compatibility with plant species (monocots vs. dicots), promoter strength and specificity, sgRNA expression efficiency, target tissue specificity, multiplex editing requirements, and vector size limitations. Coordinated high-level expression of Cas9 and sgRNA is essential for achieving high mutation efficiency [7].

Conclusion. The efficiency of CRISPR/Cas9 genome editing in plants is strongly influenced by the promoter–terminator combinations. Advances in synthetic biology have enabled the development of engineered promoters and optimized terminators, which significantly improve gene expression control. Recent studies have highlighted the importance of fine-tuning transcriptional regulation to achieve precise and efficient genome editing. Future research should focus on developing species-specific regulatory elements and minimizing gene silencing effects to further enhance the performance of CRISPR. Promoters and terminators are fundamental components of CRISPR/Cas9 plant vectors that directly influence the efficiency of genome editing. Careful selection and optimization are essential for achieving reliable and high-performance gene editing. Continued advancements in regulatory element engineering will further expand the application of CRISPR technology in plant biotechnology

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