REVIEW ARTICLE

Fidelity and Delivery: Coupled Barriers in Plant CRISPR-Cas Genome Editing

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Abstract

CRISPR-Cas platforms have transformed plant functional genomics, yet nucleases such as Cas9 and Cas12a remain constrained by two seemingly distinct limitations: imperfect target discrimination and inefficient intracellular delivery. Here, this review proposes that these constraints are functionally coupled through the intracellular abundance, nuclear access, and chromatin residence time of active Cas–gRNA complexes. Off-target activity arises from guide–target mismatch tolerance during R-loop formation, whereas rigid plant cell walls constrain the delivery of RNP and DNA cargo. These processes can become coupled when delivery limitations alter the concentration or duration of active Cas–gRNA exposure. When inefficient delivery is addressed through sustained or elevated nuclease expression, it can increase cumulative active Cas–gRNA exposure and may consequently increase off-target risk. Evidence from maize, rice, wheat, and carrot systems is synthesized and convergent biochemical solutions, including transgene-free RNP delivery, are outlined spanning herbaceous and woody species.

Keywords: R-loop formation kinetics, off-target mutagenesis, ribonucleoprotein delivery, nuclear localization signal, prime editing.

1 Introduction

Since its adaptation from bacterial adaptive immunity, the CRISPR-Cas system has become a widely used platform for targeted mutagenesis, allele engineering, and transcriptional regulation in plants, enabling rapid functional dissection of complex traits across model and crop species [1]. Cas9 and its orthologs achieve target specificity through Watson-Crick base pairing between a guide RNA and genomic DNA, followed by PAM recognition and progressive R-loop formation; however, this same biochemical mechanism permits cleavage at imperfectly matched genomic loci, generating off-target mutations whose frequency and distribution depend on guide-intrinsic mismatch tolerance and sequence context [2]. In rice and maize, such off-target events have been mapped to homologous gene family members and loci differing by as few as one to three nucleotides from the intended target, underscoring that catalytic promiscuity, not merely computational guide design, governs specificity outcomes [3, 4].

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Plant cells present a distinctive extracellular barrier as a rigid cell wall, which is absent from mammalian cells. The rigid cellulosic cell wall impedes direct entry of Cas-gRNA ribonucleoproteins and plasmid DNA, historically causing Agrobacterium-mediated transformation, particle bombardment, or protoplast-based delivery, each with distinct efficiency and genotype-dependence trade-offs [5]. Because reduced delivery efficiency is frequently compensated by prolonged or elevated nuclease expression, delivery limitations and off-target fidelity are not independent variables but are mechanistically linked through the effective dose and nuclear residence time of active Cas-gRNA complexes; attenuating Cas9 expression has been shown to simultaneously preserve on-target efficiency while suppressing off-target activity, directly supporting this coupling [6]. This review focuses only on the catalytic and cell-biological biochemistry underlying these coupled constraints, excluding computational or algorithm-guided design frameworks, and considers the emerging potential of transgene-free RNP delivery for both herbaceous and woody plants, while recognizing that efficient delivery and plant regeneration remain major constraints in woody species [7].

2 Biochemistry of Target Recognition and Off-Target Generation

Target discrimination by CRISPR-Cas nucleases is governed by a directional, kinetically staged DNA-unwinding process rather than by simple thermodynamic base-pairing complementarity. Upon PAM recognition, the Cas9-guide RNA complex nucleates a short seed duplex and propagates R-loop formation unidirectionally from the PAM-proximal to the PAM-distal end of the protospacer, a zipping mechanism in which mismatches stall strand invasion locally and compete kinetically with R-loop collapse [8]. Structural and single-molecule studies show that Cas9 interrogates DNA through discrete conformational intermediates and that PAM-proximal mismatches impose stronger barriers to productive R-loop formation and cleavage than PAM-distal mismatches, although the outcome depends on mismatch identity, position, local sequence context, and mismatch combinations, allowing the ribonucleoprotein to achieve stable binding at some partially matched loci; progression from binding to cleavage depends on the mismatch pattern and conformational activation pathway [9]. Cryo-electron microscopy has resolved this process structurally, demonstrating that full R-loop zipping triggers HNH domain docking into the target strand (Figure 1(a)); mismatch-tolerant activation under incomplete PAM-distal hybridization has been inferred from complementary biochemical and structural studies but is not directly depicted in this matched-complex structure pair [9].

The sequence determinants underlying this asymmetric mismatch tolerance are not uniform across the guide length; systematic decomposition of specificity data reveals that off-target propensity depends on combinatorial, position-dependent interactions between guide and target mismatches rather than a simple additive penalty per mismatch, explaining why some multi-mismatch off-target sites are cleaved as efficiently as perfect matches [2]. In planta, this biochemical property has direct genomic consequences: whole-genome resequencing of CRISPR-edited rice and maize lines has identified off-target mutations at loci differing from the intended target by only one to three nucleotides, frequently within paralogous gene family members sharing extended PAM-distal homology [3, 4].

Cas12a diverges mechanistically from Cas9 in ways that reshape this fidelity landscape. Rather than employing two independent nuclease domains (HNH and RuvC), Cas12a channels both non-target and target strands sequentially through a single RuvC active site, a process requiring substantial domain flexibility during R-loop propagation from a short 5-base-pair seed region [10]. Cryo-EM snapshots show that the non-target strand is only pulled into the RuvC catalytic core once R-loop formation is nearly complete, after which an α-helical lid element within RuvC guides the spatially distant target strand toward the same catalytic core for sequential cleavage [11]. This lid must reset conformationally between cleavage events, occluding the active site and requiring re-activation, a kinetic checkpoint absent from the Cas9 mechanism [10, 11]. Figure 1(b) illustrates the bookend states of this pathway, showing the initial 5-bp seed-interrogation complex and the final target-strand-in-active-site complex that together frame the conformational transition preceding lid resetting. Activation of this catalytic cycle further depends on concerted allosteric movement of the bridge helix and adjacent RuvC-II helix, indicating that Cas12a fidelity is governed by a multi-step conformational relay rather than by DNA duplex energetics alone [12].

A further biochemical layer specific to the eukaryotic

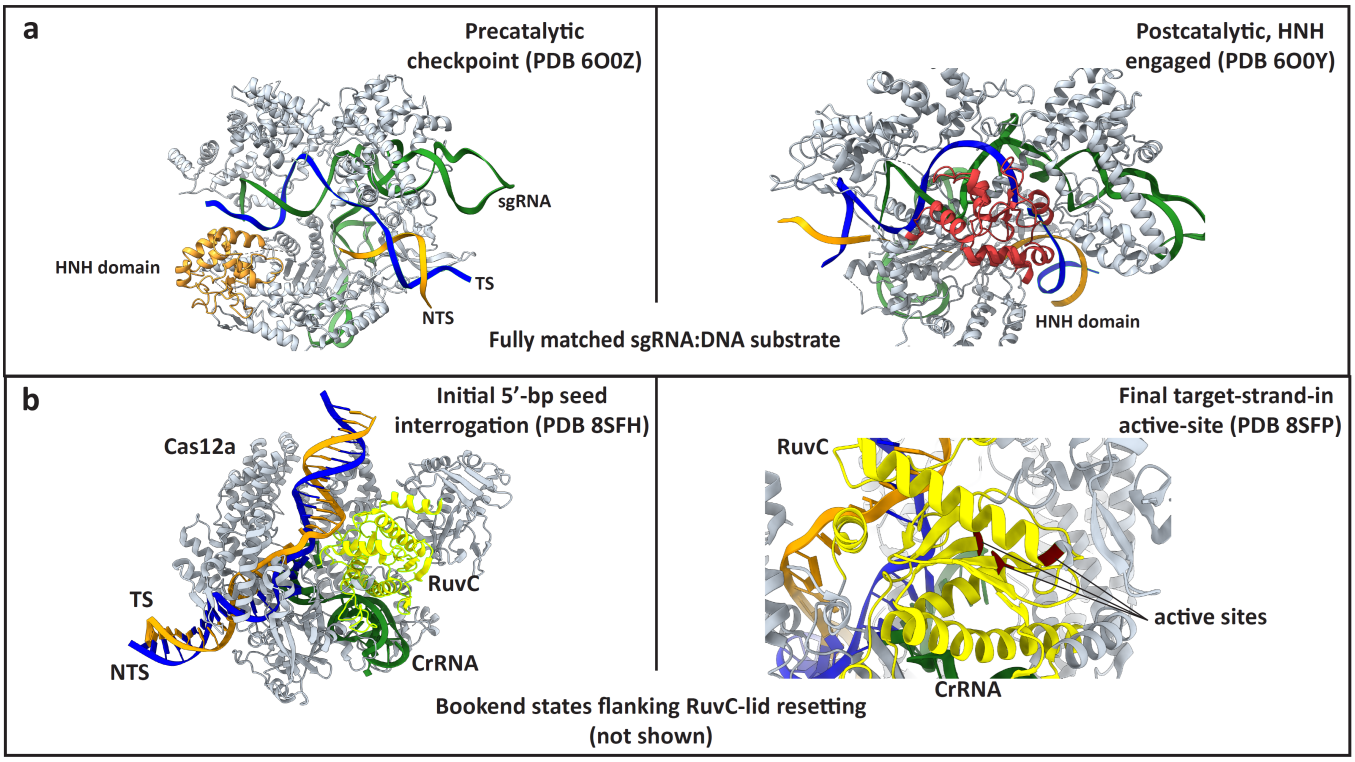

Figure 1. Structural, kinetic, and delivery determinants that couple fidelity and efficiency in plant CRISPR-Cas editing. (a) Cas9 R-loop completion promotes HNH-domain docking. Matched cryo-EM views show the *Streptococcus pyogenes* Cas9–sgRNA–DNA complex in its precatalytic checkpoint state (left; PDB 6O0Z) and postcatalytic HNH-engaged state (right; PDB 6O0Y). The HNH nuclease domain is highlighted in gold and red, respectively; single guide RNA (sgRNA), target DNA (TS), and non-target DNA (NTS) are forest green, blue, and orange. The arrow denotes the approximately 34 Å HNH repositioning. (b) Cas12a progression from seed interrogation to target-strand catalysis. Structures of the *Acidaminococcus sp.* Cas12a–crRNA–DNA complex depict an initial 5-bp RNA:DNA seed-interrogation complex (left; PDB 8SFH) and a final target-strand-in-active-site complex (right; PDB 8SFP). Cas12a is light grey; RuvC domain is in yellow, and crRNA, TS, and NTS are forest green, blue, and orange, respectively. Red sticks (D908, E993, D1263) identify the RuvC catalytic residues coordinating the scissile phosphate.

nuclear genome is chromatin occlusion. Using a nucleosome-resolution mismatch inhibition assay, Cas9 was shown to access on-target sequences less efficiently when the PAM lies buried within a nucleosome, whereas exposed mismatched targets positioned at nucleosome edges were bound with paradoxically higher affinity than nucleosome-occluded on-target sites [9]. This finding indicates that nucleosomal positioning does not uniformly suppress off-target activity but can redistribute it, favoring accessible off-target loci over inaccessible cognate targets, a phenomenon with potential implications for chromatin-structured and repeat-rich plant genomes that require direct testing in plant nuclei. Collectively, these structural and kinetic data establish that off-target generation in plant genome editing arises from the interplay of guide-intrinsic R-loop mismatch tolerance, nuclease-specific catalytic checkpoints, and local chromatin architecture, a mechanistic triad that must be addressed jointly rather than through guide-design optimization alone [13].

3 Delivery Biochemistry: From Cell Wall to Catalytic Site

Whereas the intrinsic catalytic fidelity of Cas9 and Cas12a once bound to chromatin has been addressed, editing outcomes in planta are equally constrained by an upstream biochemical problem: the physical and biophysical barriers that dictate how much active nuclease-guide complex ever reaches the nucleus. The plant cell wall, a cellulose-hemicellulose-pectin matrix whose composition, architecture, and physicochemical properties restrict the entry of large exogenous cargoes, including plasmid DNA and Cas–gRNA RNPs, imposes a size-exclusion limit that prevents direct diffusion of Cas9 or Cas12a ribonucleoprotein (RNP) and plasmid DNA into the cytoplasm, historically

causing enzymatic wall removal (protoplasting), physical disruption (biolistics), or biological vectoring (Agrobacterium) to achieve cargo entry [5]. This barrier is not merely a passive filter; nanoparticle-plant interaction studies show that cell wall pore size, surface charge, and the applied nanocarrier's zeta potential jointly determine translocation efficiency and that wall-loosening pretreatments such as zwitterionic ionic liquids can transiently relax pore constraints to permit passage of larger DNA-carrying micelle complexes without triggering cytotoxic wall-damage responses [14]. Engineered nanocarriers exploiting these structure-activity relationships have been shown to traverse the wall without external mechanical force, offering a biochemically tunable alternative to biolistic delivery [15, 16].

Once past the wall, the biochemical form of the cargo, RNP versus plasmid DNA, imposes distinct stoichiometric and kinetic constraints on the editing outcome. Pre-assembled Cas9 or Cas12a RNP complexes deliver a fixed, saturating stoichiometry of active nuclease at the moment of cytoplasmic entry, bypassing the transcription-translation delay inherent to plasmid-based expression and avoiding the risk of genomic integration of donor DNA sequences. Polyethylene glycol-mediated delivery of gRNA:Cas9 RNPs to carrot protoplasts demonstrates that simultaneous delivery of multiple pre-formed RNPs achieves multiplexed editing efficiencies reaching 71%, with defined deletion products between paired cut sites, a stoichiometric precision that plasmid-based transient expression, dependent on variable promoter strength and copy number, cannot readily replicate [17].

Nuclear entry constitutes a further, largely independent kinetic checkpoint. Cas9 and Cas12a lack intrinsic nuclear tropism and rely on fused nuclear localization signal (NLS) peptides recognized by the importin-alpha/beta heterodimer at the nuclear pore complex; the rate of this importin-mediated recognition and translocation step, rather than cytoplasmic RNP concentration alone, can become rate-limiting for effective nuclear accumulation [18]. The number and configuration of NLS motifs can alter Cas-derived editor activity; however, the magnitude of these effects is construct- and host-context dependent [19]. Beyond import-signal architecture, expression-level determinants such as intron-mediated enhancement of Cas9 transcription have also been shown to modulate editing efficiency in plants [20]. Direct quantitative comparisons of NLS-dependent import kinetics and steady-state nuclear Cas–gRNA concentrations in plant cells remain scarce; this gap limits attribution of editing outcomes specifically to nuclear import rather than to upstream delivery, expression, or regeneration variables.

Following nuclear entry, the effective editing window is further bounded by intracellular degradation kinetics. Because RNP complexes lack a genetic template for replenishment, their nuclear residence is governed by proteolytic clearance, meaning that transient, biolistically delivered Cas9-gRNA complexes in wheat shoot apical meristems achieve heritable, integration-free mutations only within a finite temporal window before the active complex is degraded, in contrast to stably integrated transgenic constructs that continuously regenerate nuclease expression at the cost of prolonged off-target exposure [21]. Promoter and sgRNA expression-cassette optimization studies in cotton further demonstrate that even at the transcriptional level, the achievable steady-state concentration of guide RNA, not merely Cas protein abundance, directly scales mutation efficiency, underscoring that delivery biochemistry and catalytic engagement are governed by the same rate-limiting supply of intact, correctly localized Cas-gRNA complex [22]. Collectively, these findings establish that cell-wall permeability, cargo stoichiometry, nuclear import kinetics, and intracellular half-life constitute sequential, mechanistically coupled checkpoints that jointly determine the effective dose of catalytically competent Cas-gRNA reaching the plant genome.

4 Convergent Solutions to the Shared Fidelity-Delivery Bottleneck

If catalytic promiscuity during R-loop propagation and dose-limiting delivery kinetics are mechanistically coupled, then effective solutions must act on the same coupled variable: the effective concentration and residence time of the catalytically competent Cas-gRNA complex at the target locus, rather than on either arm in isolation.

Protein engineering of the Cas9 scaffold itself directly addresses the catalytic side of this coupling: SpCas9-HF1 introduces alanine substitutions at non-specific DNA-contacting residues, reducing genome-wide off-target events to below detection limits by GUIDE-seq while preserving on-target activity for the majority of tested guides [23].

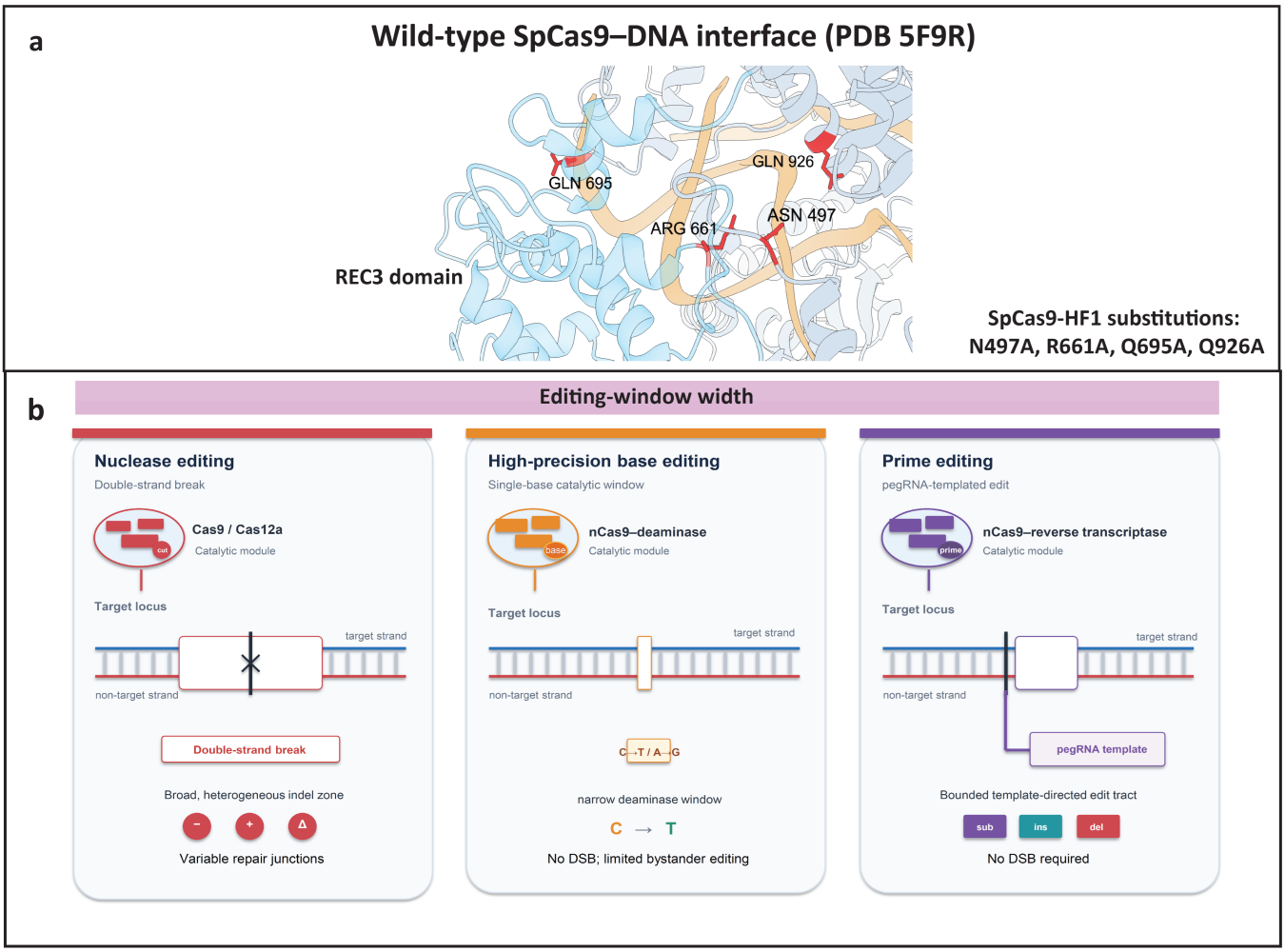

Figure 2. Convergent fidelity-engineering and editing-window strategies in plant CRISPR-Cas systems. (a) Structural rationale for SpCas9-HF1 fidelity engineering. A close-up of the active *Streptococcus pyogenes* Cas9–sgRNA–DNA complex (PDB 5F9R) shows the four wild-type residues modified in SpCas9-HF1 (Asn497, Arg661, Gln695, and Gln926; red sticks) at the protein–DNA interface. The REC3 domain is shown in pale cyan for context. The adjacent inset lists the corresponding alanine substitutions, designed to weaken non-specific phosphate-backbone contacts. (b) Editing-window geometry across CRISPR precision-editing platforms. Linear target-DNA schematics, drawn to a common scale, compare the catalytic footprint and principal repair or editing outcome of three platforms. Wild-type Cas nuclease cleavage generates a double-strand break (DSB) and a broad, heterogeneous indel zone. High-precision base editors restrict deaminase activity to a narrow, guide-positioned single-nucleotide window, enabling base conversion with reduced bystander editing. Each schematic labels the catalytic module (Cas9/Cas12a, nCas9-deaminase, or nCas9-reverse transcriptase) and target locus, with editing outcomes tagged as substitution (sub), insertion (ins), or deletion (del) where applicable. Prime editors, using a nickase Cas9–reverse transcriptase fusion directed by an engineered pegRNA, specify a bounded, template-directed edit tract that permits defined substitutions, insertions, or deletions without DSB formation. (–, +, Δ) represent heterogeneous indel repair.

However, systematic profiling of high-fidelity variants such as HiFi Cas9 and LZ3 reveals a guide-dependent efficiency trade-off, in which roughly one-fifth of single-guide RNAs suffer significant activity loss when paired with these variants, owing to variant-specific mutations in the REC3 domain that interact with non-seed guide positions 15 to 18 [24]. This trade-off indicates that fidelity engineering shifts, rather than eliminates, the specificity-efficiency boundary, a relationship most intuitively represented by mapping the four HF1-relevant substitution sites onto the wild-type SpCas9–DNA interface, with the corresponding alanine substitutions summarized alongside (Figure 2(a)).

Chemical modification of the guide RNA itself offers an orthogonal, delivery-compatible route to the same coupled variable. Substitution of ribonucleotides with 2′-fluoro or locked nucleic acid (LNA) nucleotides at

defined guide positions increases nuclease resistance and prolongs functional guide lifetime without compromising cleavage kinetics, and selectively modified crRNAs have been shown to reduce off-target cleavage in vitro relative to unmodified guides [25]. Because chemically stabilized guides extend the functional half-life of the RNP complexes identified as a rate-limiting, degradation-bound resource, this modification class effectively lengthens the productive editing window per delivered dose rather than increasing dose itself, a distinction with direct relevance to minimizing off-target accumulation over time [26, 27].

Delivery-side solutions converge on the same logic by maximizing the fraction of catalytically active complexes reaching the nucleus per unit exposure. In vitro pre-assembled RNP delivery by electroporation or PEG-mediated protoplast transfection bypasses transcriptional amplification, providing a fixed, non-renewing stoichiometric pulse of active Cas–gRNA complex that is cleared by proteolysis after a finite window, an approach that could be paired with a fidelity-engineered protein for finite-dose delivery, although quantitative on-target purity for this specific combination has not been systematically reported in plant systems. In carrot protoplasts, this strategy using pre-assembled Cas9 RNPs enabled multiplex editing with overall editing effectiveness up to 71%, although such efficiency cannot be generalized across species, target loci, or regeneration systems [17]. Nanoparticle-mediated and viral vector-based RNP delivery further extend this principle past the protoplast bottleneck, enabling wall traversal without enzymatic cell wall removal while preserving the finite-dose pharmacokinetics that limit off-target exposure [15, 16].

Two further solution classes act upstream and downstream of the catalytic step itself. Chromatin-targeting fusion domains, exemplified by dCas9-KRAB constructs that induce highly localized H3K9 trimethylation and reduced accessibility at a single targeted enhancer with minimal genome-wide off-target chromatin changes, demonstrate that chromatin state can be programmably modulated with a specificity approaching that of catalytic editing itself, suggesting an analogous strategy could be adapted to transiently open heterochromatin-occluded targets identified without increasing nuclease dose [28]. Finally, base and prime editors sidestep the double-strand-break-dependent off-target mechanism entirely: high-precision cytidine base editors engineered with shortened deaminase-Cas9 linkers achieve narrowed single-nucleotide editing that substantially reduces adjacent bystander cytidine conversion, while prime editing systems using engineered pegRNAs achieve up to 66.7% precise substitution, insertion, or deletion efficiency in rice without generating double-strand breaks or requiring donor DNA templates [29, 30]. The narrowed catalytic geometry of these editors relative to wild-type nuclease-mediated cleavage is best conveyed through a side-by-side schematic of editing-window width across nuclease, base-editor, and prime-editor platforms (Figure 2(b)). Collectively, these five convergent strategies (fidelity-engineered proteins, chemically stabilized guides, finite-dose RNP delivery, programmable chromatin modulation, and narrowed-window editors) each act on a distinct node of the same fidelity-delivery continuum, providing complementary rather than redundant routes to resolving the shared molecular bottleneck.

5 Concluding Remarks

Across the biochemical continuum examined here, from PAM-distal mismatch tolerance during Cas9 R-loop propagation [9] and the single RuvC active-site cleavage cycle of Cas12a [10], through the cell-wall permeability barrier governing RNP and plasmid entry [5], nuclear import kinetics dictated by NLS architecture, and the transient exposure achieved by DNA-free or transient delivery formats [17, 21], a unifying principle emerges: editing fidelity and delivery efficiency are two expressions of the same underlying variable, the effective dose and residence time of the catalytically active Cas-gRNA complex at chromatin. Convergent solutions spanning high-fidelity nuclease engineering [23], chemically stabilized guides [25], finite-dose RNP delivery [17], programmable chromatin modulation [28], and narrowed-window base and prime editors [29] each act on a distinct node of this continuum rather than in isolation.

Translating these mechanistic insights into field-deployable crop editing pipelines will require standardized, biochemistry-first benchmarking, quantitative R-loop kinetics, cell-wall permeability assays, and dose-response profiling, applied consistently across monocot and dicot species, ploidy levels, and tissue types, to enable direct cross-study comparison and accelerate rational selection of fidelity-delivery solution combinations for specific genomic and agronomic contexts.

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Conflicts of Interest

Ali Movahedi served as an Editor-in-Chief of the *Plant Innovation Journal* at the time of manuscript submission. To ensure the integrity of the peer-review process, Ali Movahedi was not involved in the editorial handling, peer review, or decision-making process for this manuscript, which was handled independently by another editor.

AI Use Statement

The author declares that no generative AI was used in the preparation of this manuscript.

Ethical Approval and Consent to Participate

Not applicable.

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