Agriculture and Biology 2026, 2(2), 52–74.
https://doi.org/10.70737/rse20h76

Review

Genome Editing Technologies in Plant miRNA Research: From Functional Dissection to Precision Engineering

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CITATION

Gao X., Zhang X., Yue J., Dong N., Li Y., Li J. Genome Editing Technologies in Plant miRNA Research: From Functional Dissection to Precision Engineering. Agriculture and Biology. 2026, 2(2): 52–74. https://doi.org/10.70737/rse20h76

ARTICLE INFO

Received: 17 July 2026
Accepted: 26 August 2026
Available online: 1 September 2026

Abstract: MicroRNAs (miRNAs) are endogenous small non-coding RNAs of ~21 nucleotides that serve as central regulators of post-transcriptional gene expression in plants. Unlike protein-coding genes, *MIRs* are short non-coding sequences with distinct structural characteristics that pose unique challenges for gene editing, including phenotypic redundancy, low editing efficiency in adenine and thymine-rich (AT-rich) regions, and difficulties in assessing off-target effects. Genome editing technologies, particularly the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system, have become indispensable tools in plant molecular breeding and functional genomics. In plant miRNA research, these technologies enable precise regulation of miRNA genes and their targets, facilitating the exploration of miRNA functions in plant growth, development, and stress responses. This review discusses the transformative role of genome editing in plant miRNA research, covering key applications from functional characterization of miRNA genes to engineering of miRNA-mediated regulatory tools, and further explores the potential of novel CRISPR/Cas tools for precise miRNA editing along with the growing trend of integrating gene editing with synthetic biology and multi-omics approaches.

Keywords: CRISPR/Cas; functional genomics; gene expression regulation; genome editing; non-coding sequences; plant miRNA; precision breeding

1. Introduction

MicroRNAs (miRNAs) constitute a major class of regulatory small RNAs with a canonical length of 20–24 nucleotides (nt). In plants, miRNAs influence the expression of more than 60% of genes and play pivotal roles in nearly all essential cellular processes, including cell differentiation, development, gene silencing, and stress responses [1–5]. By guiding ARGONAUTE (AGO)-containing RNA-induced silencing complexes (miRISCs) to complementary target transcripts, mature miRNAs regulate gene expression primarily through target mRNA cleavage and, to a lesser extent, translational inhibition. Compared with animal miRNAs, plant miRNAs generally exhibit higher complementarity with their target sequences, enabling highly specific recognition and regulation of downstream genes [6,7].

Plant miRNAs are encoded by *MIR* genes, which possess several unique characteristics distinct from protein-coding genes. Most *MIR* loci are relatively short non-coding genomic regions that generate characteristic stem-loop precursor structures [8,9]. During canonical miRNA biogenesis, *MIR* genes are transcribed by RNA polymerase II to produce primary miRNA transcripts (pri-miRNAs), which are subsequently processed by the DICER-LIKE1 (DCL1)–HYPONASTIC LEAVES1 (HYL1)–SERRATE (SE) microprocessor complex into precursor miRNAs and mature miRNA/miRNA* duplexes

[10]. Following HUA ENHANCER1 (HEN1)—mediated methylation and stabilization, mature miRNAs are incorporated into ARGONAUTE1 (AGO1)—containing miRISCs, which recognize complementary target transcripts and execute gene regulation (**Figure 1**).

Plant miRNAs have been demonstrated to function as critical regulatory hubs controlling a wide range of developmental and physiological processes. Conserved miRNA families, including miR156, miR159, miR166 and miR172, regulate fundamental developmental programs such as phase transition, flowering, leaf polarity and organogenesis [11–17]. For example, the miR156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) and miR172-APETALA2 (AP2) regulatory modules form conserved genetic circuits controlling juvenile-to-adult transition and reproductive development [18,19]. Other miRNA families participate extensively in environmental adaptation through modulation of hormone signaling, reactive oxygen species metabolism and stress-responsive transcriptional networks. Stress-associated miRNAs, such as miR398, miR393 and miR408, contribute to plant tolerance against drought, salinity, nutrient imbalance and oxidative stress by adjusting downstream regulatory pathways [20]. In crop species, miRNA-mediated regulatory networks also influence agronomically important traits, including plant architecture, grain size, yield formation, fertility and disease resistance [21–23].

Despite extensive progress in understanding plant miRNA biology, functional characterization of *MIR* genes remains challenging. Unlike protein-coding genes, *MIR* loci are often extremely short, structurally compact and frequently exist as members of highly redundant gene families. In addition, individual miRNA molecules may regulate multiple target genes, while closely related miRNA family members can exhibit overlapping functions, making it difficult to distinguish specific regulatory contributions. Several conventional strategies have been developed to manipulate miRNA abundance or activity, including miRNA overexpression, target mimics (MIM) and short tandem target mimics (STTM). These approaches have provided valuable insights into miRNA functions; however, they primarily regulate miRNA abundance or activity at the RNA level rather than directly modifying endogenous genomic sequences. Consequently, these methods may introduce ectopic expression patterns, incomplete suppression, or unpredictable effects caused by transgene insertion. Moreover, they are generally insufficient for dissecting the contribution of individual nucleotides within miRNA precursors, mature miRNA sequences, or miRNA-binding sites.

The emergence of genome editing technologies has fundamentally changed the landscape of plant miRNA research by enabling direct manipulation of endogenous *MIR* loci and miRNA regulatory elements. CRISPR/Cas-based approaches allow researchers to generate precise loss-of-function mutations, delete miRNA precursor regions, modify miRNA-target recognition sites, and engineer regulatory sequences in their native genomic contexts. Beyond conventional knockout strategies, the development of base editing and prime editing technologies has further expanded the capability of miRNA research toward single-nucleotide resolution and quantitative modulation of miRNA activity. These advances provide unprecedented opportunities to investigate the molecular logic of miRNA regulation and to engineer desirable agronomic traits through precise modification of endogenous regulatory networks.

In this review, we summarize recent advances in genome editing technologies applied to plant miRNA research. We first compare traditional approaches for miRNA functional analysis and discuss their limitations, followed by an overview of CRISPR-based

strategies for miRNA knockout, target-site editing and regulatory manipulation. Particular attention is given to emerging precision editing technologies, including base editing and prime editing, as well as miRNA-mediated regulatory engineering. Future opportunities for combining genome editing with synthetic biology and multi-omics approaches are also considered, with an emphasis on precise manipulation of plant regulatory networks.

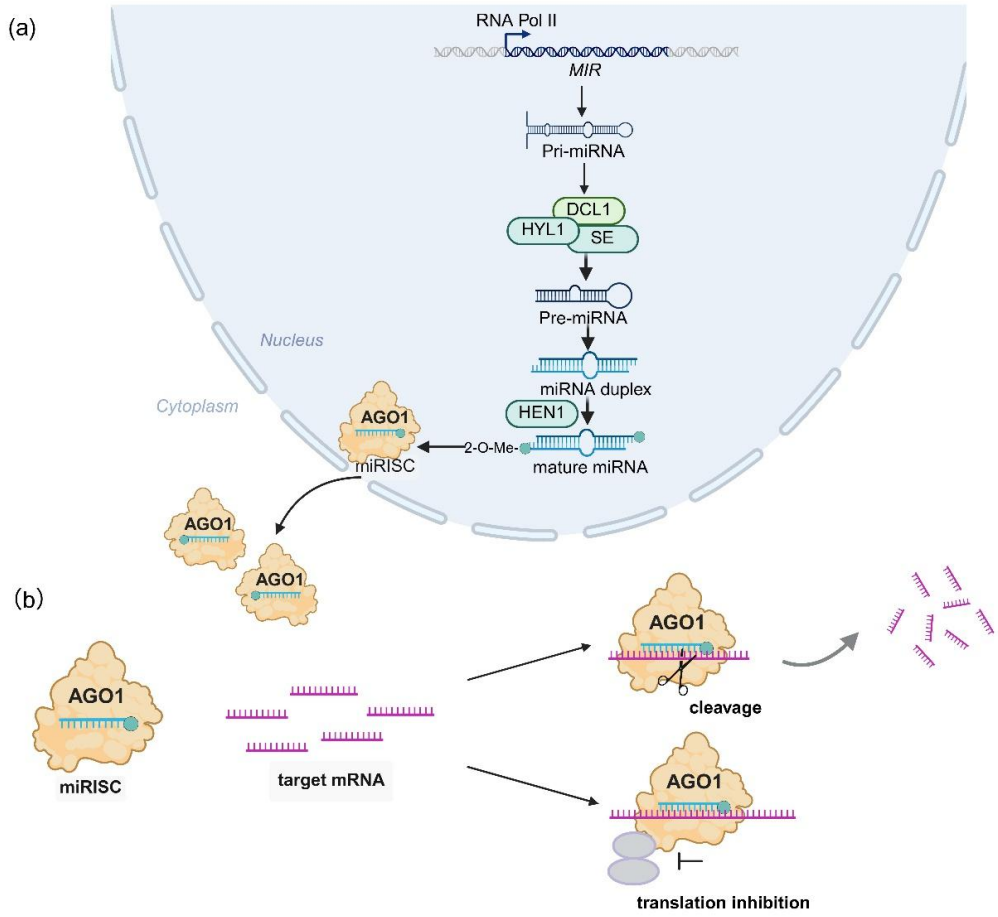


Figure 1. An overview of microRNA biogenesis and function in plants. (**a**) *MIRs* are transcribed by RNA polymerase II (RNA Pol II) to produce primary miRNAs (pri-miRNAs). In the nucleus, pri-miRNAs are sequentially processed by a microprocessor complex composed of DCL1, HYL1, and SE to generate precursor miRNAs (pre-miRNAs) and subsequently miRNA/miRNA* duplexes. The duplexes are methylated at their 3′ termini by HEN1 to enhance stability and are then exported to the cytoplasm. In the cytoplasm, the mature miRNA is loaded into AGO1 to form the miRNA-induced silencing complex (miRISC). (**b**) The AGO1-containing miRISC recognizes target mRNAs through sequence complementarity. Depending on the degree of complementarity, the mature miRNA directs either endonucleolytic cleavage of the target mRNA or translational inhibition, thereby regulating post-transcriptional gene expression. DCL1: Dicer-like 1 (an RNase III enzyme responsible for miRNA precursor processing); HYL1: Hyponastic Leaves 1 (a double-stranded RNA-binding protein involved in miRNA processing); SE: Serrate (a zinc-finger protein assisting the microprocessor complex); HEN1: Hua Enhancer 1 (a methyltransferase methylating miRNA duplexes); AGO1: Argonaute 1 (the core component of the miRISC, executing miRNA-mediated regulation).

2. Conventional Strategies for Plant miRNA Functional Characterization

Before the widespread application of genome editing technologies, plant miRNA

functions were mainly investigated through approaches that altered miRNA abundance or activity. Major strategies included miRNA overexpression, target mimicry approaches such as MIM and STTM, and miRNA target identification and validation. These methods have greatly facilitated the discovery of miRNA regulatory networks and provided important insights into the roles of miRNAs in plant development and stress responses.

2.1. miRNA Overexpression

The most straightforward strategy to investigate miRNA function is to overexpress full-length miRNA precursors driven by strong constitutive promoters such as CaMV 35S [24]. Excess mature miRNAs accumulate and trigger the cleavage or translational repression of all corresponding target genes, recapitulating loss-of-function phenotypes of downstream regulatory networks (**Figure 2**). In rice, miR397 overexpression enlarges grain size and increases panicle branch number, whereas miR319 overexpression broadens leaf blades and improves cold tolerance [25]. Similarly, constitutive upregulation of miR160, miR398 and miR7695 activates plant defense pathways and improves bacterial leaf spot resistance [25,26].

Constitutive overexpression suffers from two major drawbacks. First, if a single miRNA family targets multiple genes involved in divergent biological pathways, ectopic overexpression often induces pleiotropic defects including growth retardation and sterility [27,28]. Second, miRNA abundance far exceeds physiological levels, making it difficult to distinguish direct regulatory effects from indirect disturbances of downstream gene networks. Tissue-specific or chemically inducible promoters can partially alleviate these artifacts, yet they cannot resolve the fundamental limitation: overexpression introduces exogenous transgene copies rather than modifying endogenous *MIR* loci in situ.

2.2. Loss-of-Function Approaches via Target Mimicry: MIM and STTM

Two generations of transcript-based target mimicry tools have been established to specifically inhibit endogenous miRNA activity. Both systems exploit an endogenous regulatory mechanism: non-coding transcripts bind miRNAs through complementary binding sites and block miRNA association with authentic target mRNAs (**Figure 2**).

Target Mimic technology was developed in 2007, inspired by *Arabidopsis IPS1* gene, which naturally suppresses miR399 activity via a partially complementary binding site carrying a three-nucleotide central bulge [29]. Synthetic MIM constructs replicate this structure: artificial transcripts with miRNA-complementary sequences and a central bulge bind target miRNAs without being cleaved, leading to miRNA sequestration and functional inhibition [30,31]. This technology has been applied across multiple plant species, and large-scale MIM libraries have been constructed for conserved miRNA families in *Arabidopsis* [31]. However, MIM cannot fully suppress highly abundant miRNAs, leaving residual miRNA activity that produces weak or ambiguous mutant phenotypes [32].

Short Tandem Target Mimic was developed to overcome MIM limitations [33]. STTM transcripts contain two miRNA binding motifs separated by a 48–88 nt stem-

loop spacer, which improves structural stability and miRNA binding affinity [34,35]. Compared with MIM, STTM achieves more complete and sustained miRNA silencing and has become the dominant loss-of-function tool for plant miRNA research, with successful applications in *Arabidopsis*, rice, maize, tomato and alfalfa [33,36,37]. Importantly, STTM enables functional analysis of entire miRNA families, a task difficult to achieve via random mutagenesis due to the compact size of *MIR* loci. Nevertheless, similar to MIM, STTM relies on stable transgene transformation and cannot modify endogenous *MIR* loci, retaining all inherent drawbacks of RNA-level manipulation methods.

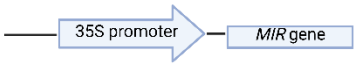
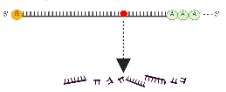
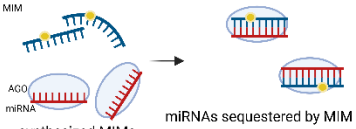
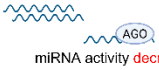
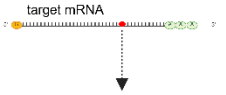
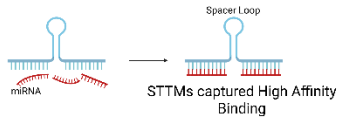
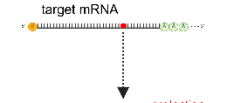
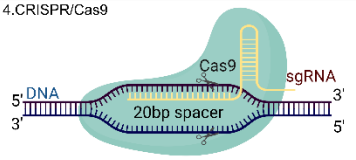
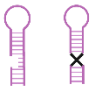
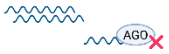
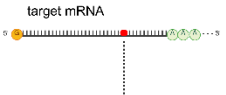
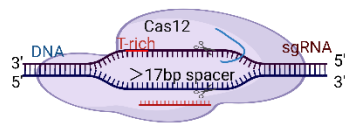
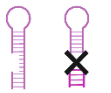
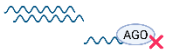
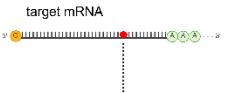
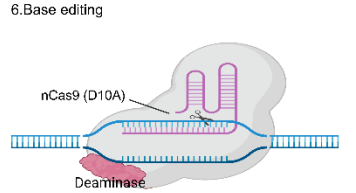
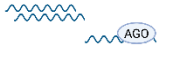
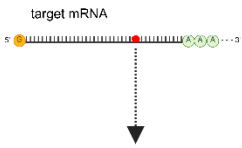
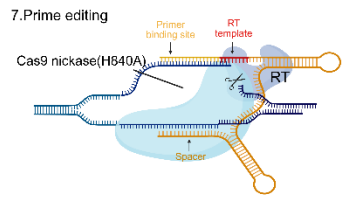
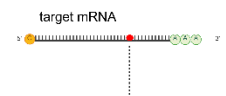
Methods	Strategy/ Mechanism	Impact on miRNA (form hairpin structure)	mature miRNA level/ activity	Impact on target mRNA (outcome)
1.Overexpression（traditional） 	a.Extra copies of *MIR* gene b.Strong promoter drives higher transcription and processing	 Usually unchanged	miRNA level/activity increase 	target mRNA  Stronger cleavage/repression
2.MIM（traditional） 	a.Synthetic target mimic with central bulge b.Binds miRNA and without cleavage c.Sequesters miRNA and inhibits its function	 Exogenous mimic (not from hairpin)	miRNA level ≈  miRNA activity decrease	target mRNA  Protection from cleavage, higher mRNA level (residual activity remains)
3.STTM（traditional） 	a.Target mimic with mismatches at cleavage site b.Prevents binding of miRNA to real target	 Usually unchanged	miRNA level ≈  miRNA activity decrease	target mRNA  Protection from cleavage, higher mRNA level
4.CRISPR/Cas9 	a.Knockout *MIR* gene (deletion/insertion) b.Mutate miRNA sequence/seed	 Disrupted or mutated hairpin	miRNA level decrease/lost  miRNA activity decrease/lost	target mRNA  Regulation released, mRNA increases
5.CRISPR/Cas12a(Cpf1) 	a.Knockout *MIR* gene b.Delete regulatory elements c.Mutates miRNA sequence	 Disrupted or mutated hairpin/regulatory elements	miRNA level decrease/lost  miRNA activity decrease/lost	target mRNA  Regulation released, mRNA increases
6.Base editing 	a.dCas9 fused with base editor b.Converts single bases in miRNA/target sites without DSBs	 Subtle base change,hairpin largely maintained	miRNA level adjusted/decrease  miRNA activity adjusted/decrease	target mRNA  Fine-tuned regulation (decrease/ increase/no change)
7.Prime editing 	a.Cas9 nickase-RT with pegRNA b.Precise edits (substitutions/insertions/ deletions) without DSBs	 Precise rewrite, structure maintained or optimized	miRNA level adjusted precisely/ decrease/increase  miRNA activity adjusted precisely/ decrease/increase	target mRNA  Precisely regulation (decrease/ increase/no change)

Figure 2. Strategies for modulating miRNA function and their effects on target mRNAexpression. **Overexpression:** Extra exogenous *MIR* gene copies driven by strong promoters (e.g., 35S) enhance miRNA transcription and processing. Mature miRNA abundance and activity rise, strengthen cleavage and repression of target mRNAs; the precursor hairpin structure remains largely intact. **MIM (Target Mimicry):** A synthetic target mimic containing a central bulge associates with miRNA-loaded AGO complexes and avoids cleavage itself. By sequestering AGO-bound miRNAs, it prevents these effector complexes from accessing endogenous target mRNAs and dampens miRNA-mediated silencing. Consequently, target-mRNA cleavage is repressed, resulting in elevated accumulation of target mRNA. **STTM (Short Tandem Target Mimic):** High-affinity synthetic mimics with mismatches at cleavage sites capture miRNAs and inhibit their binding to native targets. Precursor hairpin structure and mature miRNA quantity are unaltered; miRNA activity is drastically suppressed, reducing target mRNA cleavage and elevating target transcript accumulation. **CRISPR/Cas9:** Indels disrupt *MIR* mature sequences or stem-loop regions to generate gene knockouts. Mature miRNA abundance and activity decrease or disappear entirely, releasing target mRNAs from transcriptional repression and increasing target expression levels. **CRISPR/Cas12a (Cpf1):** Analogous to Cas9, Cas12a mediates *MIR* knockout, regulatory element deletion or miRNA sequence mutation. Disruption of precursor hairpins or regulatory regions reduces or eliminates mature miRNA activity, leading to target mRNA derepression. **Base editing:** A Cas9 nickase [nCas9 (D10A)] fused to a deaminase domain (base editor) introduces single-base conversions in miRNA precursors or target sites without double-strand breaks (DSBs). This results in subtle hairpin modifications (largely maintaining precursor structure) and adjusted mature miRNA levels/activity, enabling fine-tuned regulation of target mRNA expression (decrease, increase, or no change). **Prime editing:** Cas9 nickase (H840A) fused to reverse transcriptase (RT) and paired with a prime editing guide RNA (pegRNA) enables precise edits (substitutions, insertions, deletions) in *MIR* genes without DSBs. The miRNA precursor hairpin is either maintained or precisely modified, allowing for highly specific adjustment of mature miRNA levels and activity, leading to predictable, fine-tuned regulation of target mRNAs.

2.3. Identification and Validation of miRNA Target Interactions

Accurate identification of miRNA target genes is essential for understanding miRNA-mediated regulatory networks and interpreting phenotypic consequences caused by miRNA perturbation. Early studies mainly relied on computational prediction approaches based on sequence complementarity between mature miRNAs and target transcripts. Several prediction tools, including TargetFinder and psRNATarget, have been widely used in plants due to the high degree of complementarity between plant miRNAs and their targets [38,39]. However, computational predictions often generate false-positive candidates and require experimental validation.

Transcriptome analysis has been increasingly combined with miRNA expression profiling to identify potential regulatory relationships. The integration of small RNA sequencing and RNA-seq enables researchers to identify genes whose expression changes are negatively correlated with miRNA abundance. Nevertheless, transcriptome-based approaches cannot directly distinguish primary miRNA targets from indirect downstream effects.

Degradome sequencing, also known as parallel analysis of RNA ends (PARE), provides a powerful high-throughput approach for experimentally identifying miRNA-directed cleavage events in plants. Because plant miRNAs typically guide AGO-mediated cleavage of target transcripts, the resulting 5’ cleavage fragments can be captured and sequenced to determine precise miRNA target sites at a genome-wide scale [40]. Large-scale degradome analyses have revealed extensive miRNA–target

regulatory networks in Arabidopsis, rice, and other plant species, providing valuable resources for functional characterization of conserved and species-specific miRNA functions [40–42].

Following candidate identification, experimental validation methods, including 5’ RNA ligase-mediated rapid amplification of cDNA ends (5’ RLM-RACE), reverse transcription–quantitative polymerase chain reaction (RT-qPCR), and reporter assays, are commonly used to confirm direct miRNA–target interactions [43]. The combination of computational prediction, transcriptome analysis, degradome sequencing, and molecular validation provides a comprehensive framework for dissecting miRNA regulatory networks. Such approaches are particularly important for genome editing studies, as they allow accurate interpretation of downstream effects caused by modification of endogenous *MIR* loci or miRNA regulatory sequences.

Although conventional strategies have greatly advanced the characterization of plant miRNA functions, they still possess several inherent limitations. miRNA overexpression and target mimicry-based approaches mainly alter miRNA abundance or activity without modifying endogenous *MIR* loci or miRNA regulatory elements. Such approaches may not fully reproduce the native spatiotemporal expression patterns of miRNAs and can introduce indirect effects caused by ectopic expression or incomplete suppression. In addition, these methods are often insufficient for distinguishing the specific contributions of individual members within highly conserved miRNA families or for precisely dissecting sequence-dependent functions of mature miRNAs and their target recognition sites.

In parallel, identification and validation of miRNA targets represent fundamental and indispensable steps in plant miRNA research. Integrated approaches, including computational prediction, high-throughput sequencing-based analyses, and experimental validation, have facilitated the discovery of miRNA–target relationships and provided critical evidence for understanding regulatory mechanisms. Although these conventional strategies have substantially advanced plant miRNA studies, their applications also depend on the availability of appropriate experimental systems and cannot fully resolve the complex regulatory relationships among miRNAs, their targets, and associated biological processes.

3. Genome Editing Strategies for Functional Dissection of Endogenous Plant miRNAs

The limitations of conventional miRNA manipulation approaches have driven the development of genome editing strategies that enable direct modification of endogenous *MIR* loci and their regulatory elements. Unlike methods that alter miRNA abundance or activity at the RNA level, genome editing allows precise investigation of miRNA functions within their native genomic contexts, providing new opportunities to dissect the roles of individual *MIR* genes, regulatory regions, and miRNA–target interactions. In this section, we summarize the major genome editing strategies used for functional dissection of endogenous plant miRNAs and discuss their applications, advantages, and limitations.

3.1. *MIR* Gene Knockout by CRISPR/Cas Systems

The most direct application is to disrupt mature miRNA sequences or delete the complete precursor hairpin to abolish miRNA function. Single sgRNAs introduce small insertions or deletions (indels) within mature miRNA or miRNA* sequences, which disrupt DCL1-mediated precursor processing or AGO1 loading. By contrast, dual sgRNAs flanking the stem-loop region enable full excision of the precursor, reliably ablating miRNA function by eliminating all sequences required for miRNA biogenesis. The latter strategy is particularly critical when small indels fail to fully inactivate compact *MIR* loci, a common risk stemming from the minimal structural footprint of miRNA genes. The unique structural properties of *MIR* loci—AT-rich composition, short sequence length, and absence of protein-coding open reading frames—have driven the development of diversified CRISPR effector systems beyond canonical streptococcus pyogenes Cas9 (SpCas9). Each platform exhibits distinct strengths and limitations for miRNA-focused editing workflows. Early sequence-specific nucleases including zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), while largely superseded by CRISPR systems for routine applications, also demonstrated the feasibility of editing miRNA loci and remain valuable for orthogonal validation where CRISPR targeting is suboptimal [44–47].

CRISPR/Cas9. CRISPR/Cas9 remains widely used for *MIR* knockout and targeted mutagenesis of miRNA recognition sites. Genomic analysis in rice showed that 93.92% (556/592) of annotated miRNAs contain at least one accessible NGG protospacer adjacent motif (PAM), supporting broad applicability [23]. Editing efficiencies of 48–89% have been reported in rice T0 lines for individual miRNA genes (*OsMIR408*, *OsMIR528*) and multi-member families ("*osa-miR815a/b/c*", "*osa-miR820a/b/c*"), with similar efficiencies documented in soybean (*gma-miR1514*, *gma-miR1509*) and *Arabidopsis* (*ath-miR169a*, *ath-miR827a*) [48,49]. CRISPR/Cas9 has been used to generate loss-of-function mutations in multiple *MIR* genes, including *MIR535*, *MIR827*, and *MIR168a*, facilitating the functional characterization of these miRNAs in stress responses and plant development. In *Arabidopsis*, a dual-guide RNA strategy successfully generated *ath-miR160a* null mutants carrying targeted fragment deletions, with higher editing efficiency than the single-guide RNA approach.

A key practical advantage of Cas9 is its capacity to induce large-fragment deletions via dual sgRNAs, which reliably generates complete loss-of-function alleles by removing the entire miRNA hairpin and avoids residual miRNA activity frequently observed with small indels [50]. However, the strict NGG PAM requirement limits target accessibility in the AT-rich genomic landscapes characteristic of most plant *MIR* loci. Additionally, small indels within mature miRNA sequences occasionally produce hypomorphic alleles retaining partial processing and regulatory activity, complicating phenotypic interpretation.

TREX2-FrCas9. The TREX2-FrCas9 system was developed to enhance large-fragment deletion at miRNA loci. Fusion with the TREX2 exonuclease increases deletion size without compromising editing efficiency, producing deletions predominantly longer than 11 bp (peak at 16–20 bp)—substantially larger than the 6–13 bp typical of Cas12a [51,52]. This profile reduces the risk of functional rescue from

small in-frame indels and ensures near-complete miRNA inactivation. Multiplex editing of the complex *OsMIR156j* locus using four sgRNAs efficiently generated heritable mutants, validating TREX2-FrCas9 as a powerful addition to the plant miRNA editing toolkit.

CRISPR/Cas12a (Cpf1). Cas12a has emerged as a complementary effector uniquely suited for miRNA editing, primarily owing to its recognition of T-rich 5'-TTTV-3' PAM sequences that are abundant within AT-rich *MIR* loci [53,54]. Additional advantages include generation of 5' staggered overhangs (which may facilitate homology-directed repair), smaller effector protein size, shorter crRNAs (~43–60 nt), and an extended recognition–cleavage distance that supports larger deletions. For miRNA knockout, Cas12a predominantly produces deletions of 6–13 bp (peak at 8–9 bp), which efficiently disrupt mature sequences and hairpin structures; single crRNAs have achieved deletion efficiencies of up to 100% at certain miRNA loci, reliably generating null alleles [55]. Orthologs including AsCas12a, FnCas12a, and LbCas12a have been successfully applied in rice, soybean, tobacco, tomato, and maize [56–58], and the autonomous crRNA processing capability of Cas12a simplifies multiplex editing workflows. However, in certain plant systems, the transformation efficiency and biallelic mutation rate of Cas12a are generally slightly lower than those of Cas9; ongoing protein engineering studies are currently focused on addressing this limitation.

A research team at Southwest University compared the efficiency of Cas12a and Cas9 in knocking out the rice non-coding gene *OsMIR390* and confirmed that Cas12a is more suitable than Cas9 for effectively knocking out miRNA genes and generating reliable loss-of-function mutants [55]. Using this system for genome-wide miRNA knockout in rice, they identified multiple loss-of-function mutants related to developmental processes, grain traits, and quality, providing technical support for large-scale, systematic miRNA functional studies.

3.2. miRNA Target Site Editing

Rather than disrupting the miRNA itself, this strategy introduces sequence variants at miRNA-binding sites located within target mRNA coding regions or 3' untranslated regions (3' UTRs) to eliminate miRNA-mediated repression. Plant miRNAs generally exhibit near-perfect complementarity with their target transcripts. Therefore, even a single nucleotide substitution at a critical pairing position can disrupt miRNA-mediated regulation of a specific target without affecting other transcripts [59,60]. Compared with *MIR* knockout strategies, miRNA target-site editing provides a more precise approach to dissect individual miRNA–target regulatory relationships. By introducing specific mutations into endogenous miRNA recognition sequences within target transcripts, this strategy selectively disrupts a single miRNA–target interaction while preserving endogenous miRNA expression and other regulatory pathways. Therefore, target-site editing enables direct evaluation of the contribution of specific targets to miRNA-associated phenotypes.

The importance of miRNA target sites has been demonstrated in several conserved regulatory modules. For example, alterations in the miR319 recognition site within *TCP* transcripts affect miRNA-mediated repression and lead to abnormal leaf development in *Arabidopsis*, highlighting the functional importance of target-site sequences [61].

Similarly, the miR165/166–*HD-ZIP III* regulatory module demonstrated that precise miRNA–target pairing is required for proper organ development and polarity regulation [62].

Although target-site editing offers higher specificity than *MIR* knockout, its application in plants remains challenging. Accurate identification of functional targets, potential redundancy among miRNA family members, and the difficulty of editing short recognition sequences represent major limitations. Future integration of high-confidence target identification methods and precision genome editing technologies will facilitate more systematic analysis of miRNA regulatory networks.

3.3. Regulatory Region Editing

In addition to editing *MIR* loci or miRNA target sites, genome editing can be used to dissect the cis-regulatory regions that control *MIR* transcription. Promoter editing provides a means to investigate how individual regulatory elements contribute to gene expression and to generate allelic variation without altering the encoded product. Importantly, rather than producing a simple on/off phenotype, targeted modification of promoter regions can generate a continuum of expression states, thereby providing a useful strategy for investigating dosage-dependent biological functions and quantitative traits. In rice, a CRISPR/Cas12a promoter editing (CAPE) system was developed to efficiently generate quantitative trait variation by targeting key regions of promoters. Editing of *OsGBSS1*, *OsGS3*, and *OsD18* promoters produced graded phenotypic variation in grain starch content, grain size, and plant height, respectively, demonstrating that promoter editing can convert cis-regulatory variation into quantitative agronomic traits [63].

Beyond generating quantitative variation, cis-regulatory editing enables functional dissection of promoter architecture by generating defined regulatory alleles with distinct transcriptional and phenotypic outputs. Systematic modification of promoter regions has demonstrated that individual cis-regulatory elements can contribute differentially to gene expression and developmental processes, providing a means to resolve the functional organization of complex regulatory regions [64]. More recently, Prime Editing-mediated Promoter Engineering (PEPE) was developed in rice to generate tiled, overlapping deletions across the 1.8-kb *D53* promoter. Different promoter deletions produced distinct transcriptional outputs, with some deletions reducing *D53* expression by 70–85%, whereas a distal deletion increased expression 2.2-fold, demonstrating that promoter architecture can be systematically dissected to achieve quantitative control of endogenous gene expression [65]. Together, these studies highlight the potential of promoter and cis-regulatory editing to generate tunable expression phenotypes through precise manipulation of regulatory sequences.

Although such promoter engineering approaches have not yet been extensively applied to *MIR* loci, they provide a promising framework for future manipulation of *MIR* regulatory regions. Because *MIR* genes are subject to complex transcriptional and post-transcriptional regulation, precise modification of their regulatory regions could potentially enable quantitative modulation of endogenous miRNA expression while preserving the native genomic context. Such strategies could therefore complement *MIR* knockout by generating graded and context-dependent changes in miRNA activity, providing an additional approach for dissecting dosage-dependent miRNA

functions.

To systematically recapitulate the current landscape of genome editing applications in plant miRNA research and clearly distinguish experimentally validated strategies from prospective approaches, we summarize representative published studies in **Table 1**.

Table 1. Summary of representative genome editing studies in plant miRNA research.

Plant Species	miRNA/MIR Locus or Target Site	Editing Tool	Editing Strategy	Major Functional Outcome	Validation Status	Reference
Oryza sativa	miR156a/miR396c/miR528 target sites (knocked into 3′UTR of GID1 etc.)	CRISPR/Cas9	Target-site knock-in (MiRKD)	Conditional gene knockdown	Validated	[66]
Oryza sativa	*OsMIR390* and 9 other uncharacterized OsMIRNA genes	CRISPR/Cas12a (LbCas12a)	knockout	Grain and seed developmental functions	Validated	[55]
Oryza sativa	miR396 target site (*OsGS2/GRF4* coding region)	CRISPR/Cas9	Target-site editing	Increased grain weight and yield	Validated	[67]
Solanum tuberosum	3 miRNA loci (dual-sgRNA strategy)	CRISPR/Cas9	expression fine-tuning	Mutation type and allelic dosage modulate miRNA abundance	Validated	[68]
Solanum lycopersicum	*MIR164a/MIR164b/MIR164d*	CRISPR/Cas9	knockout	miRNA family functional specialization	Validated	[69]
Oryza sativa	miR396 target sites (*OsGRF4/OsGRF8* coding regions)	CRISPR/Cas9	Target-site editing	Grain size and brown planthopper resistance	Validated	[60]
Oryza sativa	*MIR396e/MIR396f*	CRISPR/Cas9	knockout	Grain size, panicle branching, and yield; enhanced yield gain under nitrogen-deficient conditions	Validated	[70]
Oryza sativa	13 miRNA genes (including *OsmiR818b*)	CRISPR/Cas9 (rCas9)	knockout	miRNA abundance and drought response	Validated	[71]

4. Precision Genome Editing Technologies for miRNA Sequence and Functional Engineering

The previous section summarized major CRISPR-based strategies for miRNA manipulation. This section focuses on the shift from binary loss-of-function approaches toward quantitative and nucleotide-level analysis enabled by base editing and prime editing. These technologies make it possible to examine not only whether a miRNA is functional, but also how its expression level, seed pairing, and precursor structure influence its regulatory effects.

4.1. Base Editing for Single-Nucleotide Resolution of miRNA Functions

Cytosine and adenine base editors mediate precise C•G-to-T•A and A•T-to-G•C conversions, respectively, without introducing double-strand breaks [72]. For miRNA research, this technology is uniquely suited to introducing single-nucleotide changes in seed regions, mature miRNA sequences, or miRNA-binding sites on target mRNAs—alterations that can redirect or fine-tune miRNA–target interactions with minimal collateral damage. Base editing efficiencies in plants range from 15% to 75%, with indel formation typically below 1%, ensuring high product purity. The high precision of base editors makes them particularly valuable for dissecting the functional contribution of individual nucleotides within miRNA regulatory sequences and for mimicking natural sequence polymorphisms associated with desirable traits [73].

However, conventional base editors are restricted to a limited range of transition mutations, primarily C•G-to-T•A conversions mediated by cytosine base editors (CBEs) and A•T-to-G•C conversions mediated by adenine base editors (ABEs), which constrains their application for introducing diverse nucleotide variations. Recent advances in base editing technologies have expanded the editable sequence space beyond canonical C-to-T and A-to-G conversions. For example, glycosylase-based cytosine base editors (CGBEs) enable C•G-to-G•C transversions by incorporating DNA glycosylase activities into base editing systems, whereas adenine transversion editors (AYBEs) provide a strategy for generating A•T-to-C•G conversions, further broadening the spectrum of programmable nucleotide substitutions [74,75]. Although these expanded-spectrum editors provide new opportunities for precise manipulation of regulatory sequences, their applications in plants remain limited compared with conventional CBEs and ABEs, and their potential for endogenous *MIR* locus editing requires further investigation.

4.2. Prime Editing for Programmable miRNA Sequence Engineering

Prime editing represents the most versatile precision editing platform currently available. It employs a Cas9 nickase fused to an engineered reverse transcriptase, guided by a prime editing guide RNA (pegRNA) that encodes the desired edit, to mediate targeted insertions, deletions, and all 12 types of point mutations without exogenous donor DNA or double-stranded DNA breaks [76,77]. For miRNA research, prime editing delivers unparalleled resolution: it enables rational rewriting of mature miRNA sequences, reengineering of precursor hairpin architectures, and precise single-nucleotide substitution at miRNA target binding sites, supporting high-resolution functional dissection and *de novo* design of miRNA regulatory modules. Nevertheless, practical deployment of prime editing in plant miRNA research faces substantial technical barriers. Reported editing efficiencies in plants were low, and the short length of miRNA sequences provides limited space for designing the primer binding site and reverse transcription template required for stable pegRNA hybridization, which is thought to be a major factor underlying the low efficiency at miRNA loci [78]. Continuous optimization of pegRNA design, reverse transcriptase processivity and cellular delivery systems is underway to overcome these limitations. Recent efforts have focused on improving prime editing performance through optimization of reverse transcriptase activity, editor architecture, and pegRNA stability. Next-generation prime editors, including PE6 and PE7, have been developed through protein engineering and optimization of editor components, resulting in improved editing performance in experimental systems [79,80]. PE6 variants incorporate evolved and engineered reverse transcriptases with enhanced activity, whereas PE7 was developed by fusing the N-terminal RNA-binding domain of the endogenous small RNA-binding protein La to the prime editor to improve pegRNA stabilization and editing efficiency [79,80]. Importantly, plant-specific evaluation of PE6 variants has demonstrated substantial improvements in editing efficiency. In transgenic rice, PE6c, which contains an evolved and engineered reverse transcriptase derived from the yeast Tf1 retrotransposon, achieved an average editing efficiency more than 3.5-fold higher than PEmax across 18 agronomically important target sites

in 15 genes [81]. These advances indicate that continued optimization of prime editor components can substantially improve editing efficiency in plants. However, the application of advanced prime editors, particularly PE6/PE7-derived systems, to endogenous *MIR* locus engineering remains largely unexplored and requires further evaluation. The key features of these platforms are summarized below to facilitate tool selection (**Table 2**). Cas12a and TREX2-FrCas9 are optimal for complete miRNA knockout, particularly in AT-rich regions where Cas9 PAM availability is limited; Cas9 is preferred for high-throughput, family-level multiplex editing; base editors excel at single-nucleotide fine-tuning of seed regions and target sites; and genome editing technology (Prime editing) still offers unrivalled precision in rewriting sequences when ultra-precise editing is required although currently limited by low efficiency.

Table 2. Comparative overview of CRISPR platforms for plant miRNA editing.

Platform	PAM/PAM Preference	Editing Products	Primary Advantage	Key Limitations in miRNA Editing
Cas9 (SpCas9)	NGG	Small indels; large deletions with dual sgRNA	Broad targeting; dual sgRNA for complete knockout	PAM-scarce in AT-rich regions; small indels may retain function
Cas12a (Cpf1)	TTTV (T-rich)	6–13 bp deletions (peak 8–9 bp)	Ideal for AT-rich miRNA loci	Lower biallelic rates in some species
Base editors (CBE/ABE)	NGG (Cas9-based) or TTTV (Cas12a-based)	C→T or A→G transitions	Single-nucleotide precision; no DSB required	Restricted to transition mutations; no knockout
Prime editing	NGG (Cas9-based)	All substitutions, small indels, defined deletions	Unrestricted sequence rewriting capability	PAM-scarce in AT-rich regions; complex pegRNA design
TREX2-FrCas9	TA-rich palindromic sites	Large deletions (>11 bp, peak 16–20 bp)	Reliable null allele generation	Limited targeting range

4.3. Quantitative Modulation of miRNA Activity through Precision Editing

Classical knockout generates categorical loss-of-function alleles and is indispensable for verifying the absolute necessity of a miRNA in biological processes. However, such on/off perturbations cannot resolve the nuanced quantitative logic governing miRNA regulatory circuits, which depend on four critical variables undetectable via simple knockout:

(i) Expression threshold: The minimum miRNA concentration required to trigger measurable target repression in specific cell types;

(ii) Seed register offset: Single-nucleotide shifts in seed pairing drastically alter the spectrum of targeted transcripts [82,83];

(iii) Precursor structural integrity: Hairpin stability directly modulates DCL1 processing efficiency and mature miRNA yield [47];

(iv) IsomiR stoichiometry: Differentially processed miRNA isoforms (isomiRs) exhibit divergent AGO binding affinities and target preferences.

In contrast, precision editing technologies provide new opportunities to quantitatively modulate miRNA activity by introducing subtle sequence changes within *MIR* genes, miRNA processing regions, or regulatory elements.

Single-nucleotide substitutions generated by base editing can alter key regions of miRNA precursors or mature miRNA sequences, including processing sites and target recognition regions, thereby modifying miRNA accumulation, stability, or target

specificity without disrupting the entire *MIR* locus. Similarly, prime editing enables programmable introduction of defined sequence variations, providing greater flexibility for creating allelic series with different levels of miRNA activity. These precisely engineered variants can help dissect the relationship between miRNA sequence features and regulatory outputs.

Beyond sequence modification, targeted editing of *MIR* promoter regions and other cis-regulatory elements offers an additional strategy for tuning miRNA expression levels. By generating regulatory variants with altered transcriptional activity, researchers can establish a spectrum of miRNA expression states and evaluate their effects on downstream targets and plant phenotypes. Such quantitative regulation is particularly valuable for studying miRNAs involved in developmental processes and stress responses, where complete loss of function may cause severe pleiotropic effects.

Together, precision editing approaches provide a framework for moving beyond binary manipulation of miRNA functions toward quantitative and context-dependent regulation. This capability will facilitate deeper understanding of miRNA regulatory mechanisms and support the engineering of desirable traits through fine-tuning of endogenous plant regulatory networks.

5. Synthetic miRNA-Based Regulatory Modules

5.1. Artificial miRNA Systems for Targeted Gene Regulation

Artificial microRNAs represent an orthogonal silencing strategy that hijacks the endogenous miRNA biogenesis machinery to repress specific protein-coding genes, rather than inhibiting miRNAs themselves [84,85]. By replacing the native miRNA/miRNA* duplex within a conserved precursor scaffold with custom-designed sequences, amiRNAs guide RISC-mediated cleavage of any target gene with high specificity and minimal off-target effects (**Figure 3**) [86]. Optimized precursor scaffolds, such as those derived from *Arabidopsis*.

miR319a or rice *miR528*, have simplified cloning and enabled broad application across diverse species. In rice, amiRNAs designed against *Pds*, *Spl11*, and *Eui1* efficiently downregulated their targets and produced expected phenotypic changes [87]. When combined with tissue-specific or inducible promoters, amiRNAs achieve spatiotemporal gene repression superior to conventional RNA interference systems [88,89]. However, amiRNAs serve only as gene silencing tools and cannot dissect the biogenesis, processing or target spectrum of endogenous natural miRNAs. Furthermore, like all traditional tools, amiRNAs depend on random transgene insertion and cannot mediate in situ genomic modification.

5.2. MiRKD for Conditional Gene Regulation

Beyond direct editing of miRNA loci, a novel strategy termed MiRKD (miRNA-mediated in-locus knockdown) has recently been developed to realize programmable gene repression by harnessing endogenous miRNA regulatory pathways [66]. MiRKD employs CRISPR/Cas9 to insert miRNA recognition elements (target sequences) into the 3′ UTR of target genes via homology-directed repair. After integration, the edited gene becomes subject to regulation by the corresponding endogenous miRNA:

transcripts are cleaved or translationally repressed in cells and tissues where the miRNA is naturally expressed, achieving spatiotemporally patterned gene knockdown without exogenous regulatory components (**Figure 3**). In rice, three distinct regulatory modes were validated using the gibberellin receptor gene *GID1*: constitutive knockdown mediated by ubiquitously expressed *miR156a* (up to 97% repression), shoot-specific repression by *miR396c*, and long-day-dependent temporal downregulation by *miR528* (over 95% inhibition) [66]. Stable transgenic lines confirmed editing fidelity, heritable regulatory effects and minimal disturbance to endogenous miRNA homeostasis. Notably, MiRKD is not restricted to plants; proof-of-concept experiments in mammalian cells have verified its broad cross-kingdom applicability [66].

The core merit of MiRKD lies in its capacity to repurpose evolutionarily shaped native miRNA spatiotemporal specificity to achieve transgene-free conditional gene regulation. Unlike constitutive promoters or chemically inducible systems, MiRKD requires no exogenous regulatory elements and does not introduce foreign proteins. Current limitations include its exclusive function in gene downregulation, the demand for rigorous pre-screening of suitable miRNA–target pairs, and the low efficiency of homology-directed repair in most plant species. Nevertheless, MiRKD clearly demonstrates how genome editing can be combined with endogenous small RNA pathways to develop powerful regulatory tools with functions far beyond simple gene knockout.

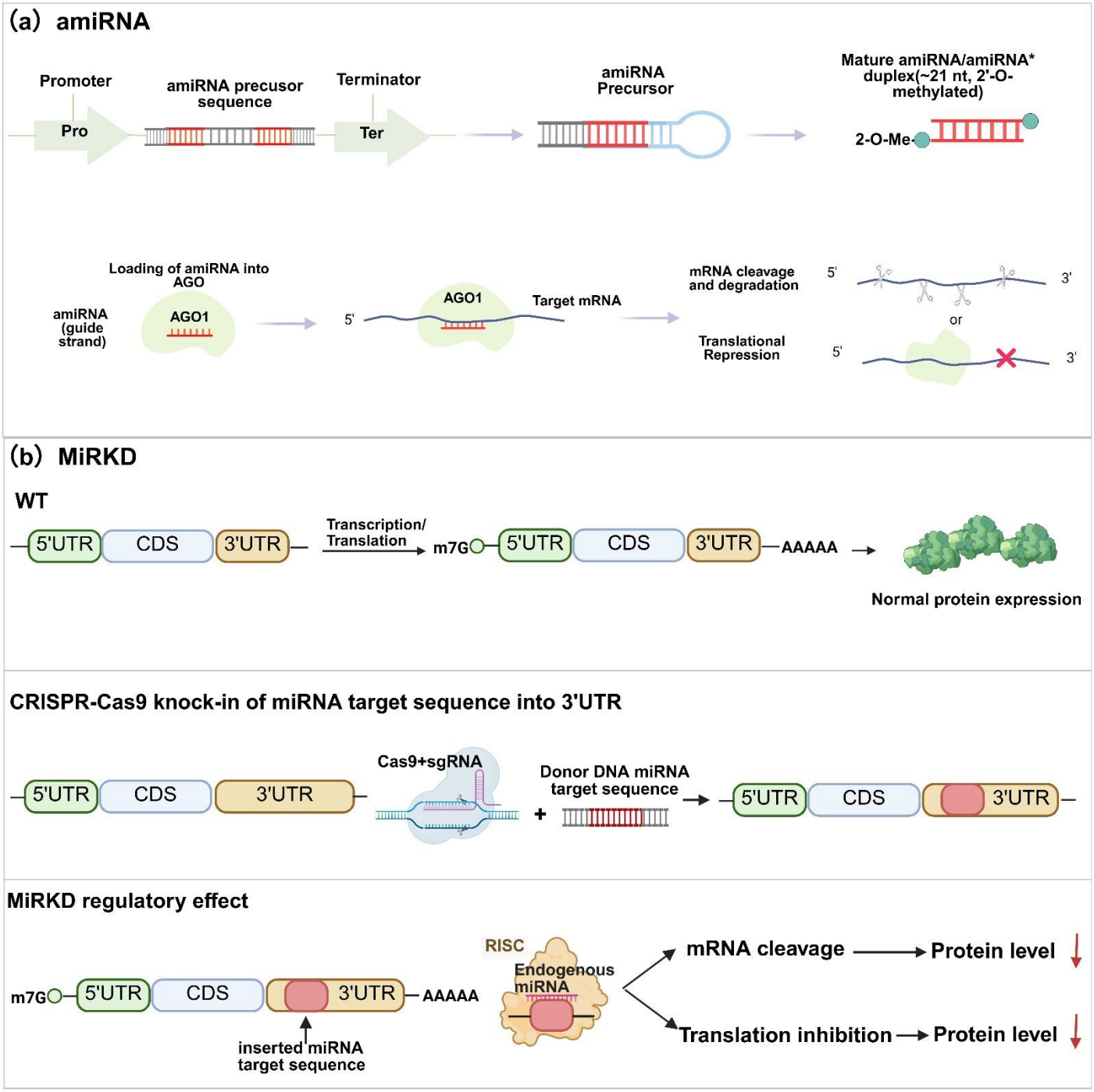


Figure 3. Schematic representation of amiRNA-mediated gene regulation and MiRKD-mediated gene knockdown. (**a**) **amiRNA-mediated gene regulation:** An engineered amiRNA precursor containing a designed target sequence is expressed from a transgene and processed into a mature amiRNA/amiRNA* duplex. The mature amiRNA is loaded into AGO1 to form an active RNA-induced silencing complex (RISC), which recognizes complementary sequences in the target mRNA through sequence-specific base pairing and induces target mRNA cleavage and degradation or translational repression, thereby reducing target gene expression. (**b**) **MiRKD-mediated gene knockdown:** In the wild-type state, the target gene is normally transcribed and translated to produce the functional protein. A specific miRNA target sequence is introduced into the 3′ UTR of the endogenous target gene through CRISPR/Cas9-mediated knock-in. The inserted target site enables endogenous miRNAs to recognize the modified transcript and recruit RISC, resulting in mRNA cleavage and/or translational repression and consequently reduced protein accumulation.

6. Future Research Perspectives

Genome editing has reshaped the landscape of plant miRNA research; however, miRNA loci present unique structural challenges that are not typically encountered in protein-coding gene editing, including AT-rich sequences that limit PAM availability, extremely short target regions that constrain editing design, widespread functional redundancy among miRNA family members, and the generally low efficiency of precision editing systems. Addressing these challenges requires coordinated advances in editing tool development, experimental strategy optimization, and multi-omics analytical frameworks. Six promising research directions are highlighted:

(i) Expanding CRISPR toolkits for AT-rich non-coding loci: develop novel Cas orthologs and SpCas9 variants with expanded targeting ranges or PAM-relaxed/PAM-independent capabilities (e.g., Cas9-NG and CasX), together with *de novo* designed nucleases optimized for targeting AT-rich miRNA loci and their complex secondary structures. (ii) Optimizing prime editing for short non-coding templates: develop dual-pegRNA architectures, stabilized hairpin pegRNA scaffolds and high-processivity thermostable reverse transcriptases to improve editing efficiency on compact miRNA sequences; leverage upgraded PE6/PE7 systems for enhanced performance [90].

(ii) Multiplex editing to resolve miRNA family redundancy: harness Cas12a's autonomous crRNA processing and polycistronic tRNA-sgRNA systems for simultaneous knockout of multiple paralogs, combined with transcriptome-wide degradome profiling to map family-specific target repertoires.

(iii) Integration of genome editing and synthetic miRNA biology: combine promoter remodeling, precursor engineering and target site editing to construct synthetic miRNA regulatory circuits responsive to developmental or environmental cues, enabling rational design of stress-resistance and flowering-time traits.

(iv) Transgene-free and DSB-free precision editing for agricultural applications: adopt RNP ribonucleoprotein delivery and viral transient expression systems to generate edited crops without foreign DNA integration; leverage base/prime editing's DSB-free nature to produce regulatory-friendly single-nucleotide agronomic alleles.

(v) Systems-level multi-omics integration: combine genome editing with small RNA-seq, degradome-seq, ribosome profiling and metabolomics to distinguish primary miRNA regulatory effects from secondary network perturbations, guiding rational multiplex trait engineering.

7. Conclusion

Diversified CRISPR genome editing platforms have substantially expanded the experimental scope of plant miRNA research, shifting experimental approaches from indirect RNA-level interference to direct, heritable, locus-specific genomic modification. This transition is driven by two key developments: the inherent technical limitations of classical overexpression, MIM/STTM and amiRNA tools, and the rapid expansion of versatile editing systems including Cas9, Cas12a, base editors, prime editors and large-fragment deletion nucleases such as TREX2-FrCas9. Three transformative conceptual advances emerge from miRNA editing research:

1. Locus-specific knockout enables separation of redundant paralog functions within multi-gene miRNA families, an experimental feat unachievable via transgenic RNA interference.
2. Single-nucleotide precision editors unlock high-resolution dissection of seed domains, precursor hairpin architectures and miRNA–target pairing rules, moving research from phenotypic observation to mechanistic nucleotide-level interpretation.
3. Innovative strategies including target site editing, promoter fine-tuning and MiRKD expand miRNA manipulation beyond simple loss-of-function knockout, enabling quantitative expression calibration and spatiotemporally conditional gene repression.

Nevertheless, the unique molecular signatures of *MIR* loci—AT richness, compact sequence space and evolutionary functional redundancy—continue to impose distinct technical barriers separating miRNA editing from protein-coding gene manipulation. Future progress relies on continuous innovation of PAM-flexible nucleases, optimized precision editors and high-capacity multiplex editing systems. The convergence of genome editing, synthetic biology, and multi-omics profiling may further extend plant miRNA research from single-locus perturbation toward systematic regulatory network analysis and engineering. Continued advances in these technologies are expected to facilitate a deeper understanding of plant non-coding RNA biology and may support their future application in precision molecular breeding and sustainable agriculture.

Author contributions: X.G., J.Y., J.L. and Y.L. conceived the manuscript framework; J.Y. and X.Z. collected and sorted literature data; X.G. and N.D. analyzed existing editing technology performance; Y.L. and J.L. supervised the whole work and revised the manuscript. All authors read and approved the final manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (Nos. 32401793, 32400335), Hefei Municipal Natural Science Foundation (No. HZR2449), the Scientific Research Project of Anhui Academy of Agricultural Sciences (No. 2026YL006).

Conflict of interest: The authors declare no conflict of interest.

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