

Cite this: *Sustainable Food Technol.*,
2026, 4, 1372

Integrating nucleic acid research and computational strategies for advancing plant food security

Yashika,^a Leena Aggarwal *^a and Pradeep Pant *^b

The evolution of plant breeding, from traditional techniques to cutting-edge omics-driven approaches (genomics, transcriptomics, proteomics, and metabolomics), has revolutionized crop yield enhancement since the mid-twentieth century. Today, genomics empowers breeders with powerful tools to directly associate genetic variation with phenotypes, accelerating the development of desirable traits. Building on this foundation, nucleic acid-based diagnostic techniques (PCR and LAMP) and gene-editing platforms (ZFNs, TALENs, and CRISPR/Cas), along with RNAi enable precise detection and manipulation of plant gene expression. This review focuses on various genome editing and diagnostic strategies for plant protection that aim to improve stress resilience, productivity, and nutritional value. Additionally, we highlight the latest computational strategies and methodologies that support the precise and rational design of such interventions with robust tools for targeted crop improvement. Looking ahead, these breakthroughs are poised to drive innovation across agriculture, biotechnology, and nucleic acid testing, opening new frontiers in sustainable food production and precision breeding.

Received 21st July 2025
Accepted 10th December 2025

DOI: 10.1039/d5fb00405e

rsc.li/susfoodtech

Sustainability spotlight

Ensuring global food security while facing climate change and population growth demands sustainable innovations in agriculture. This review highlights how nucleic acid-based diagnostics and gene-editing technologies—such as PCR, LAMP, CRISPR/Cas, and RNAi—are revolutionizing plant protection and crop improvement. By enabling precise, efficient, and targeted breeding strategies, these tools support resilient, high-yield crops and reduce dependence on chemical inputs. The integration of computational approaches further enhances the rational design of genetic interventions. This work aligns with UN SDG 2 (Zero Hunger) and SDG 13 (Climate Action) by promoting sustainable agricultural practices and developing climate-resilient crops, thus contributing to global efforts toward sustainable food production and improved nutritional outcomes.

1. Introduction

Population growth and climate change exert substantial pressure on global food security by inducing physiological and environmental stresses in plants, thereby enhancing their susceptibility to both abiotic stressors (such as drought, salinity, and temperature extremes) and biotic stressors (including pathogens, pests, and weeds).^{1,2} Biotic stressors contribute significantly to global agricultural losses, with estimated yield reductions ranging from 20% to 40%, leading to substantial economic impacts.³ According to the Food and Agriculture Organization (FAO), plant pests destroy as much as 40% of global crop yields annually, leading to economic losses exceeding USD 220 billion from plant diseases and at least USD 70 billion from invasive insect infestations.⁴ Plants

are pivotal in sustaining life on Earth, as they are primary sources of food and energy, forming the foundation of ecological and biological systems. Ensuring effective crop protection against threats is crucial for sustaining agricultural productivity and meeting the growing population demand.⁵ According to World Population Prospects 2022,⁶ the global population reached 8 billion in November 2022, and the UN's medium-variant projection indicates that it will peak at approximately 10.4 billion in 2080s.⁷ To address these challenges and to attain the second Sustainable Development Goal, *i.e.*, “zero hunger”, substantial efforts are necessary for the transition from conventional high-input agriculture toward more resilient, diversified and technologically supported production approaches.^{8–10} Over the last few decades, plant breeding and other technologies have made significant contributions toward minimizing hunger and extreme poverty.^{2,11} Prior to the genomic era, conventional breeding methods rely on controlled hybridisation and selection of desirable traits through natural processes to develop new plant varieties.¹² Unlike marker-assisted selection,

^aDepartment of Chemistry, Netaji Subhas University of Technology, Delhi-110078, India. E-mail: leena.aggarwal@nsut.ac.in

^bDepartment of Biotechnology, Bennett University, Greater Noida, India. E-mail: pradeep.pant25@gmail.com



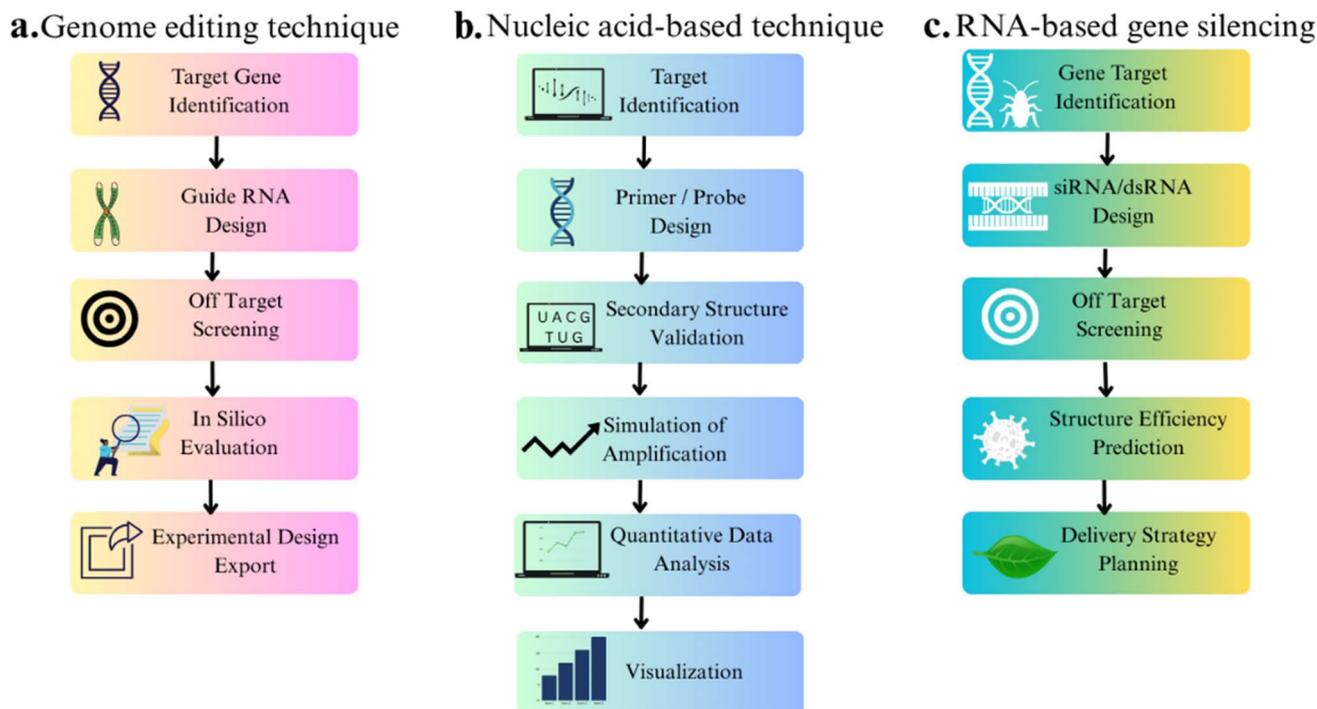


Fig. 1 (a) Genome editing workflow, starting with target gene identification, followed by gRNA or DNA-binding module design using computational tools, off-target prediction, minimization, and editing efficiency simulation. (b) Nucleic acid-based pipeline including target sequence selection, primer/probe design, structural validation, thermodynamic analysis, and *in silico* amplification for specificity and sensitivity. (c) RNA silencing process, featuring siRNA/dsRNA design via tools like Si-Fi, off-target screening, efficiency prediction, and delivery optimization.

conventional breeding is slower and less precise, making it insufficient to meet the rising global food demand.¹³ Innovative strategies to enhance crop yield and stress resistance have been transformed by genetic engineering.^{2,14} Techniques, such as Novel Plant Breeding Techniques (NPBTs) and Next Generation Sequencing (NGS), collectively accelerate crop improvement by providing genomic insights and enabling targeted manipulation of plant genetic materials. These approaches offer relevant, versatile, cost-effective, and time-efficient strategies that enhance precision in modern plant breeding.^{2,15} These techniques facilitated the development of crop plants with enhanced agronomic traits, improved nutritional quality, and increased resistance to both biotic and abiotic stresses. These technologies not only address the shortcomings of conventional breeding but also offer flexibility by enabling the use of genomic information from both model and non-model plant species.¹⁵ In addition to genetic improvements, early and accurate detection of pathogens plays a crucial role in managing plant diseases and minimizing crop loss. Nucleic acid-based diagnostic techniques such as Polymerase Chain Reaction¹⁶ (PCR), quantitative Polymerase Chain Reaction¹⁶ (qPCR), and digital Polymerase Chain Reaction¹⁷ (dPCR) enable rapid and sensitive detection of plant pathogens even at early infection stages. These techniques detect specific DNA or RNA sequences of pathogens, providing precision and timeliness in field-level diagnostics. This review critically discusses genome editing tools, nucleic acid-based diagnostic

techniques, and RNA-based technologies, along with the computational approaches that enhance their precision and applicability. A critical aspect of these technologies is the integration of computational approaches to design, evaluate, and optimize editing tools. For genome editing techniques such as Zinc Finger Nucleases¹⁸ (ZFNs) and Transcription Activator-Like Effector Nucleases¹⁹ (TALENs), software tools predict target site specificity based on DNA-binding motifs, while for Clustered Regularly Interspaced Short Palindromic Repeats²⁰ (CRISPR/Cas9), several platforms are available such as CRISPR Off-target Sites with Mismatches, Insertions and Deletion²¹ (COSMID) for designing guide RNA (gRNA) and CHaracterization and OPTimization of CHOPping tools²² (CHOPCHOP) for assisting in identifying suitable target sites and minimizing off-target effects. Likewise, nucleic acid-based diagnostic techniques such as PCR¹⁶ and dPCR¹⁷ rely on *in silico* tools for primer/probe design and target validation. These computational approaches enhance the accuracy, efficiency, and scalability of both genome editing and diagnostics, facilitating precision in plant protection and crop improvement, as shown in Fig. 1. Together, these pipelines demonstrate how computational approaches have been developed to support the design and optimization of nucleic acid-based tools, including genome editing nucleases, by enabling the prediction and reduction of off-target effects. These advancements contribute significantly to crop improvement, enhance resistance to biotic and abiotic stresses, and improve the nutritional quality of plants.



2. Literature search strategy

A comprehensive literature search was undertaken to identify studies relevant to plant genome-editing platforms, RNA-based technologies, and nucleic acid-based diagnostic approaches. The search was performed across major scientific databases, including PubMed, SciFinder and Google Scholar. Boolean operators and targeted keywords were applied, such as “CRISPR/Cas9 and plant genome editing”, “ZFNs”, “TALENs”, “RNA interference in plants”, “nucleic acid-based diagnostics”, “LAMP”, “NASBA”, “plant pathogen detection”, and “computational genome-editing tools”. Additional terms such as “RNAi delivery”, “siRNA design”, “omics-driven crop improvement”, and “biosensing technologies” were also used. Literature published between 2000 and 2024 was prioritized, with earlier foundational studies incorporated when scientifically justified.

3. Genome editing techniques

Genetic engineering has been a central area of research for several years in elucidating gene functions. This field encompasses the application of physical and biological mutagenesis, along with the identification of molecular mechanisms to enhance the crop yield.²³ It relies on utilization of engineered nucleases, which consist of sequence-specific DNA-binding domains conjugated to a nonspecific DNA cleavage module.²⁴ It includes several methods, such as ZFN,¹⁸ TALEN,¹⁹ and CRISPR/Cas9.²⁵ These are chimeric nucleases that facilitate highly efficient and precise genome editing by inducing site-specific DNA double-strand breaks (DSBs). These DSBs activate intrinsic cellular DNA repair pathways, including the

error-prone Non-Homologous End Joining (NHEJ) and the high-fidelity Homology-Directed Repair (HDR) mechanisms.²⁶

3.1. Zinc-finger nucleases (ZFNs)

ZFNs are site-specific endonucleases engineered for targeted DNA cleavage, facilitating precise genome modification. It consists of two distinct domains: a zinc finger domain that binds to the target DNA and a nuclease domain derived from the FokI restriction enzyme, which is responsible for generating DSBs. Zinc finger domains are protein motifs that fold around one or more zinc ions and are capable of recognizing specific DNA sequences^{18,24} as shown in Fig. 2a. Zinc Finger Proteins (ZFPs) contain a tandem array of Cys2-His2 zinc fingers, each of which binds with approximately 3 base pairs (bp) of targeted DNA.^{27,28} It was reported^{18,24} that individual ZFNs used three fingers to bind a 9-bp target, which enabled ZFN dimers to specify 18 bp of DNA per cleavage site. More recent advancements^{18,24} have incorporated more fingers, and a variety of strategies have been described in the literature for designing ZFPs with new, user-chosen binding specificities.^{18,24} By designing multiple zinc finger domains, ZFNs can be engineered to target a particular DNA sequence with high precision.

The nuclease domain of FokI restriction enzyme is crucial for the function of ZFNs, as it facilitates the targeted cleavage of DNA within complex genomes.^{18,24} It must dimerize for effective cleavage of DNA.^{29,30} The monomer of FokI is catalytically inactive and its natural dimerization is weak therefore, cleavage can be achieved by constructing two sets directed to DNA sequence and joined to the cleavage domain³¹ as shown in Fig. 2b. The nuclease domain introduces DSBs in the DNA at the

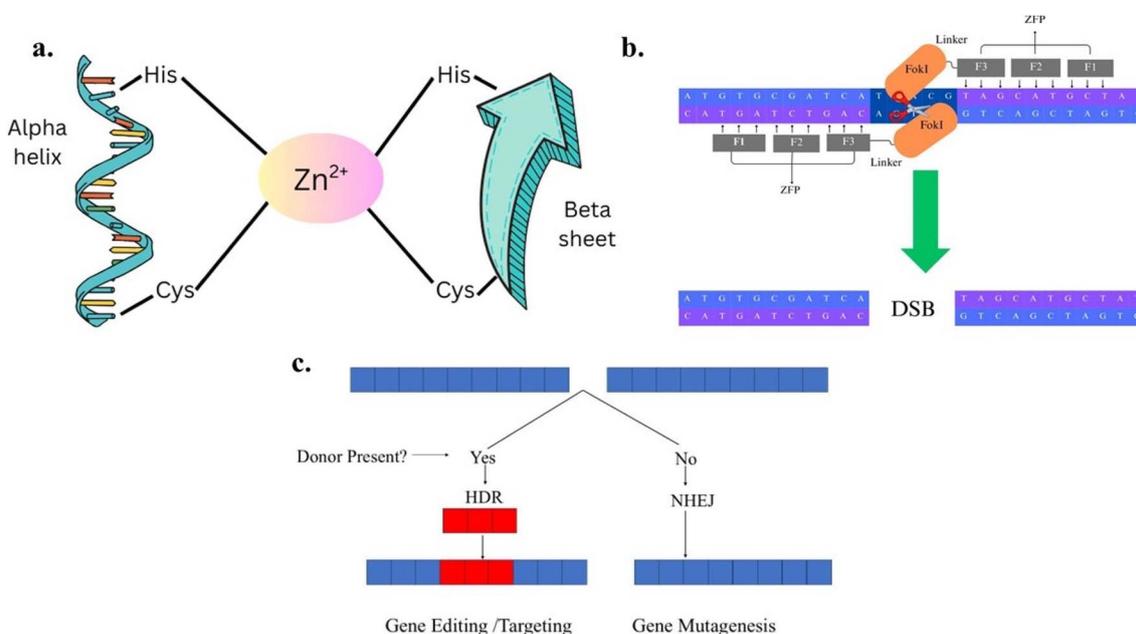


Fig. 2 Structure and mechanism of zinc finger nucleases. (a) Schematic representation of a tandem array of Cys2-His2 zinc finger protein. (b) Illustration of a pair of zinc finger nucleases binding to target DNA sequence. Zinc finger proteins are shown as grey boxes, with vertical arrows indicating base pair contacts. The FokI nuclease domain (orange) is linked to the zinc finger protein and introduces a double-strand break in DNA. (c) Different repair mechanisms resulting from the introduction of double-strand breaks in the presence and absence of donor DNA.



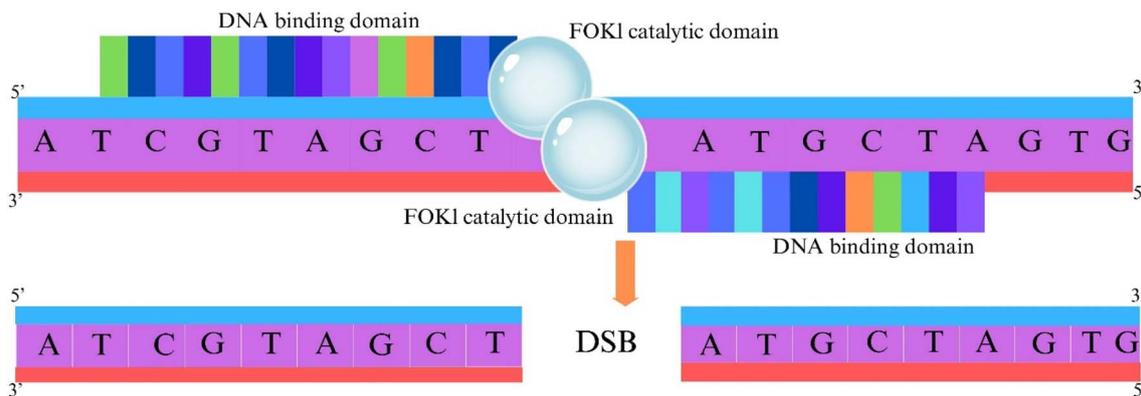


Fig. 3 Schematic representation of transcription activator-like effector nuclease architecture and double-strand break induction.

targeted site, and this break activates the repair mechanisms, which occur *via* NHEJ (absence of donor DNA) or HDR (presence of donor DNA), as shown in Fig. 2c. Targeting or editing of gene at the site of break takes place in the presence of a template donor flanking DNA gene.^{18,24} Rejoining of the two broken DNA ends takes place in the absence of template donor flanking DNA gene, with some insertion or deletion often causing mutations due to frameshifts and effectively knocking out the targeted gene at the site of break, which leads to disruption of the target gene.^{18,24}

Although ZFNs have been proven as effective technology, it also has certain drawbacks. It is an expensive process of designing protein domains for individual genes and has a risk of off-target DNA cleavage caused by faulty interactions. These challenges prompted the emergence of newer gene-editing tools, such as TALENs and CRISPR/Cas9 which offer easier construction and greater efficiency.³²

3.2. Transcription activator-like effector nucleases (TALENs)

In 2011, *Nature Methods* recognized TALENs as the Method of the Year.^{19,33} The advancement of the TALEN system technique is linked to research on bacteria of the genus *Xanthomonas*, which is pathogenic to crop plants such as rice, pepper, and tomato. These bacteria secrete effector proteins (transcription activator-like effectors, TALEs) into the cytoplasm of plant cells, modulating cellular processes and enhancing host susceptibility to infection.³² Later, it was found that these effector proteins are also capable of DNA binding and activate the expression of the target gene.³²

TALE proteins consist of a central domain responsible for DNA binding and a nuclear localization signal that facilitates their targeting of gene transcription.³⁴ The central domain is composed of repeating monomeric units, each specifically recognizing and binding a single nucleotide within the target sequence. These units are tandem repeats of 34 amino acid residues, and the last tandem repeat in the domain has 20 amino acids.³⁵ Amino acids at positions 12 and 13 are highly variable and thus called Repeat Variable Di-residues^{30,35} (RVDs). RVDs are responsible for recognition of a specific nucleotide, and the four most common RVDs are histidine–aspartic acid

(HD), asparagine–glycine (NG), asparagine–isoleucine (NI), and asparagine–asparagine (NN), accounting for each of the four nucleotides cytosine (C), thymine (T), adenine (A) and guanine (G), respectively.^{35,36} The first amino acid residue in RVDs, *i.e.* H or N, is not directly involved in nucleotide binding but plays a crucial role in stabilizing the spatial conformation of the domain. In contrast, the second amino acid residues in RVDs, *i.e.* D, G, I and N interact directly with the target nucleotide: D and N form hydrogen bonds, while I and G engage *via* van der Waals forces.^{35,37} The DNA-binding domain is integrated into a genetic construct containing a half-repeat, the N-terminal domain, and the catalytic domain of *FokI*.³⁵ It functions in pairs, with binding sites strategically positioned on opposite DNA strands and separated by a short spacer sequence (12–25 bp). Upon nuclear entry, the nucleases recognize and bind to their target sites, facilitating the dimerization of *FokI* domains at the C-terminal of the chimeric protein. This dimerization induces DSBs within the spacer sequence³² as shown in Fig. 3. DSBs activate two repair pathways in the cell *i.e.* NHEJ and HDR.³⁸

Despite the success of TALENs, a significant limitation remains as it recognizes the target site based on DNA–protein interactions due to which it leads to some fundamental structural difference which causes its low off target activity, low specificity/effectiveness, and high production cost.³⁹ These challenges prompted the emergence of a newer gene-editing tool *i.e.*, CRISPR/Cas9.

3.3. Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)

In 1987, Yoshizumi Ishino⁴⁰ and team first discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in *Escherichia coli*, characterizing them as unique DNA repeats separated by spacer sequences. In the early 2000s, Barrangou *et al.*⁴¹ worked on *Streptococcus thermophilus* and identified that bacteria containing spacer sequences homologous to bacteriophages and viral genomes exhibit immunity against infection. During viral infection, CRISPR spacers are transcribed into CRISPR RNAs (crRNAs) with Cas proteins, target and cleave viral DNA or RNA to block infection.²⁰ Emerging CRISPR-associated



nucleases such as Cas12, Cas13, and Cas14 hold significant promise for the development of transgene-free (foreign DNA free) crops.⁴² These next-generation genome editors enable precise genetic modifications, offering a regulatory advantage that facilitates easier approval and commercialization across various countries.²⁰ It is one of the best state-of-the-art genome editing technologies that is gaining popularity because of its broader applications across different organisms.^{42–44} The CRISPR–Cas system is mainly classified into two main classes as 1 and 2. Class 1 system includes type I, III and IV multiple effector proteins for the RNA-guided target cleavage, while Class 2 includes type II, V, and VI effector proteins which required only one RNA-guided endonuclease for the DNA sequence cleavage.²⁰ Type I utilizes Cas3, which acts like a molecular motor, helping to unwind and cut DNA or RNA structures. Type II employs the well-known Cas9 protein, which accurately cuts double-stranded DNA at targeted locations. Cas9 contains two domains *i.e.*, HNH domain and RuvC-like domain. The HNH domain cuts the complementary strand of crRNA, whereas the RuvC-like domain cleaves the opposite strand of double-stranded DNA.⁴⁵ Type III involves Cas10, which can target single-stranded DNA and contains a specialized region called the palm domain that enables its activity. In Type IV, the system includes a protein known as csf1, which functions in a similar way to Cas8. Type V uses Cas12 (also called Cpf1), a versatile protein capable of cleaving both single and double-stranded DNA, and it also plays a role in DNA repair. Lastly, Type VI features Cas13, which is unique because it targets RNA instead of DNA. It contains the Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domain that allows it to cleave single-stranded RNA in both simple and complex organisms.⁴⁶ The recent advent of CRISPR derived methodologies is base editing and prime editing, which have substantially expanded the scope and utility of precision genome editing.^{47,48} This review presents an overview of key developments in CRISPR/Cas technology and its applications in modern horticulture and agriculture.

The CRISPR/Cas9 system is a bacterial RNA-guided immune defense, which targets and eliminates foreign DNA from plasmids, bacteriophages and functions as a form of bacterial “immune system”.⁴⁹ It facilitates precise gene modification by introducing targeted DNA cleavage, followed by endogenous

DNA repair mechanisms.^{43,50} CRISPR/Cas9 technology, first applied in plant genome editing in 2013, has revolutionized the field by enabling precise, efficient, and versatile genetic modifications.^{51,52} CRISPR/Cas9 requires the Cas9 protein and a Protospacer Adjacent Motif (PAM) sequence for efficient DSB formation in the target DNA. Cas9 utilizes a guide RNA (gRNA) duplex composed of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) for target recognition as shown in Fig. 4a. The crRNA typically 18–20 nucleotides long, is essential for recognizing and binding to the target DNA sequence.^{53,54} The tracrRNA ranging from 50 to 150 nucleotides, plays a vital role in the Cas9-mediated DNA cleavage process.^{53,54} In current genetic engineering techniques, the duplex is combined into a single molecule called single guide RNA (sgRNA) as shown in Fig. 4b. More specifically, sgRNA is generated by linking the 3' end of crRNA with the 5' end of tracrRNA using a connector sequence.⁵⁵ The PAM sequence length varies between two and six nucleotides, such as NGG (any nucleotide (N) followed by two guanine units), NAG (any nucleotide followed by adenine and thymine units), CTT (cytosine followed by two thymine units), and TTTV (three thymine units followed by V, where V is A, C, or G). PAM functions as a key indicator for identifying target sites as it instructs Cas9 to cleave a DNA strand at a precise location.^{56,57} The seed region adjacent to PAM consisting of 10–12 base pairs determines Cas9 specificity and makes it more essential than other regions in the sgRNA.⁵⁵ The interaction allows Cas9 to make precise DSBs in the DNA.⁵⁸ DSBs then activate repair processes, which can occur through one of the two primary mechanisms NHEJ and HDR, that are then utilized to introduce genetic modifications.^{58,59} Both NHEJ and HDR provide versatility and precision in plant genome editing and enhance resistance to diseases and insect pests^{60,61} however, HDR remains challenging due to its limitations in supplying sufficient repair templates.⁶²

3.3.1 Base editing. It is an innovative technique that enables precise, programmable nucleotide changes without breaking the DNA strand or using a donor template.⁶³ It comprises two main parts: a Cas enzyme that binds to DNA in a programmable way and a single-stranded DNA-modifying enzyme that performs the specific nucleotide change. Two primary classes of DNA base editors *i.e.*, Cytosine Base Editors (CBEs) and Adenine Base Editors (ABEs) have been developed

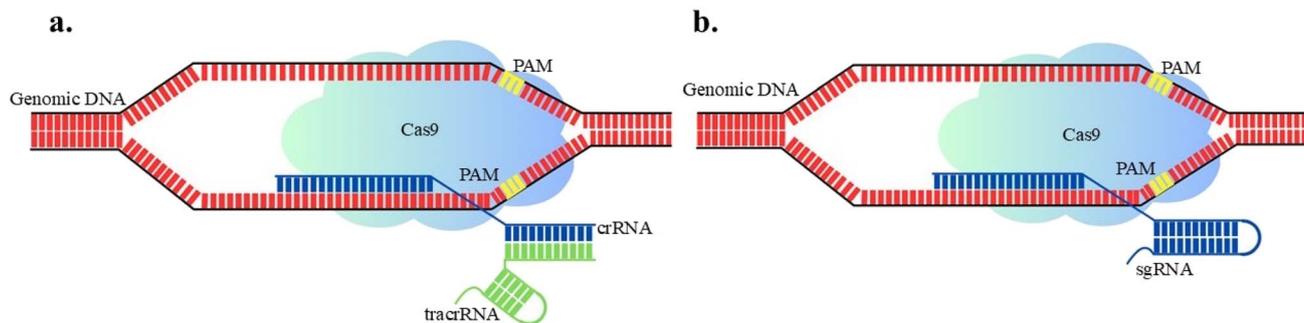


Fig. 4 Basics of the Clustered Regularly Interspaced Short Palindromic Repeats technology in genome editing. (a) Native system with Cas9 guided by CRISPR RNAs (crRNA) and trans-activating CRISPR RNA (tracrRNA). (b) Modern Clustered Regularly Interspaced Short Palindromic Repeats system using a single guide RNA (sgRNA) that fuses CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA).



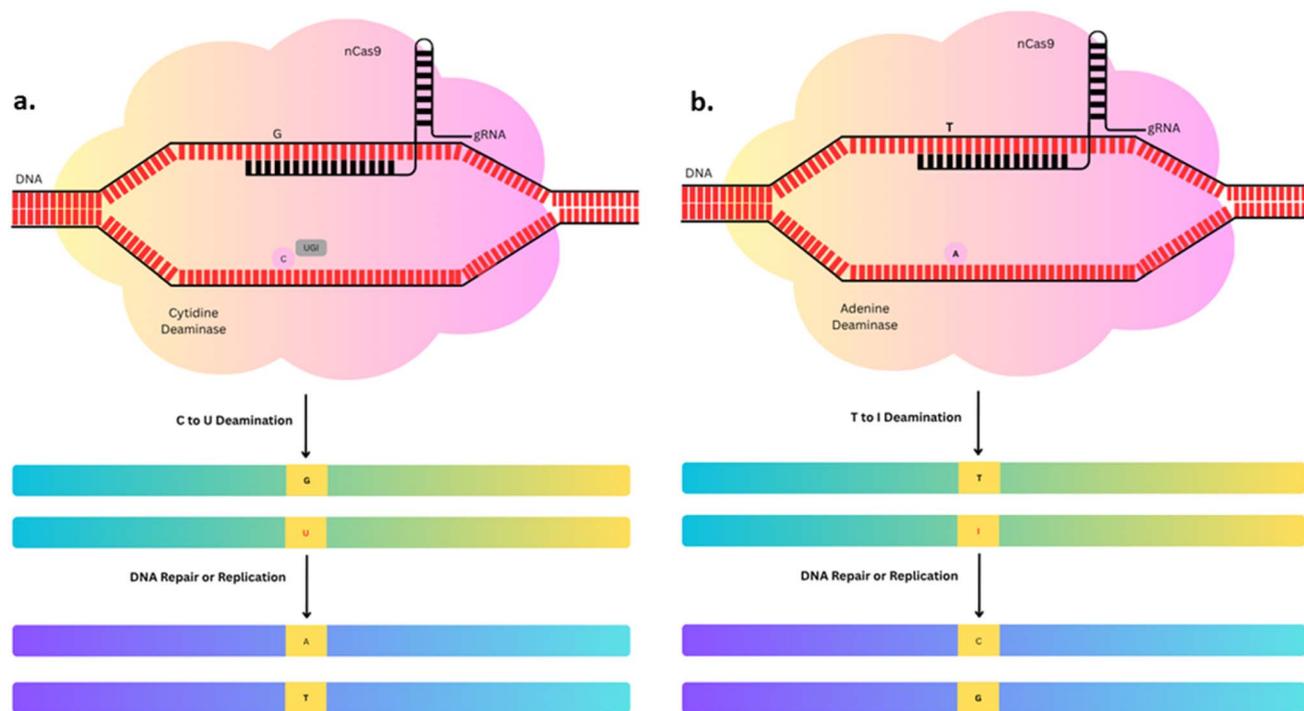


Fig. 5 Schematic illustration of the primary class of base editing tools. (a) Cytosine Base Editors (CBEs) and (b) Adenine Base Editors (ABEs).

which enable four transition mutations such as cytosine (C) to thymine (T), adenine (A) to guanine (G) and *vice versa*.⁶⁴ These advanced editors expand conventional base editing by enabling transversion mutations and multiplex nucleotide substitutions beyond the limits of single-base systems.

3.3.1.1 Cytosine Base Editors (CBEs). The first-generation CBE (CBE1) was developed by Liu and co-workers in 2016.⁶⁵ The CRISPR/Cas9-mediated base editing platform incorporating cytosine deaminase facilitates highly precise and efficient single-nucleotide substitutions at defined genomic loci without generating double-stranded DNA breaks. This approach holds substantial potential for targeted gene correction and enhancement of genetic diversity across yeast, plant, mammalian, and human cells.⁶⁶ It converts C to U (uracil), which is subsequently recognized as T during replication, resulting in a C-G to T-A transition. It has low efficiency because of cellular-mediated repair of the U-G intermediate in DNA by Base Excision Repair (BER). To overcome this, several base-editors such as CBE2 and CBE3 were developed to improve the editing efficiency. CBE3 shows the highest efficiency by six-fold over CBE2. It was developed by restoring the histidine residue at position 840 (H840) within the HNH catalytic domain of Cas9 to produce a base editor utilizing the Cas9 nickase (nCas9) variant. This introduced a single-strand nick in G containing a strand of U-G intermediate, directing the cellular repair machinery to favour conversion of the intermediate to a U-A pair, which is subsequently transformed into a stable T-A base pair during DNA replication⁶⁴ as shown in Fig. 5a.

3.3.1.2 Adenine Base Editors (ABEs). CBEs are restricted to mediating C-G to T-A transitions, which substantially limit their capacity to correct a wider set of pathogenic nucleotide

substitutions. Notably, methylated cytosines exhibit a high susceptibility to spontaneous deamination, and it is estimated that nearly half of all pathogenic point mutations could theoretically be corrected through ABEs by converting an A-T base pair back to its original G-C configuration.⁶⁷ ABEs function through a similar mechanism as CBEs. The ABE-dCas9 fusion complex binds to the target DNA sequence in a guide RNA directed manner, where the deoxyadenosine deaminase domain catalyzes the conversion of adenine to inosine. During DNA replication, inosine is recognized as guanine, leading to the substitution of the original A-T base pair with a G-C base pair at the designated genomic site⁶⁴ as shown in Fig. 5b.

In comparison to CBEs, ABEs produce markedly cleaner editing outcomes, characterized by an almost complete absence of indels and no reported instances of significant off-target A-to-G substitutions. Alkyladenine DNA Glycosylase (AAG), the enzyme responsible for recognizing and excising inosine from DNA, does not enhance ABE efficiency or product purity relative to wild-type cells.⁶⁸ CBEs and ABEs are limited to four transition mutations, a constraint that prompted the development of prime editing for broader nucleotide modification.

3.3.2 Prime editing. It is an advanced CRISPR-based genome-editing technique described by Anzalone *et al.*⁶⁹ to overcome the limitations of CBEs and ABEs, which are restricted to mediating transition mutations (C·G → T·A and A·T → G·C). Unlike conventional CRISPR-Cas9 systems that rely on DSBs and donor templates, prime editing employs a fusion of Cas9 nickase (nCas9) and reverse transcriptase (RT), guided by a prime-editing guide RNA (pegRNA)⁷⁰ as shown in Fig. 6. The pegRNA not only guides nCas9 to the target DNA site but also carries an additional sequence encoding the desired



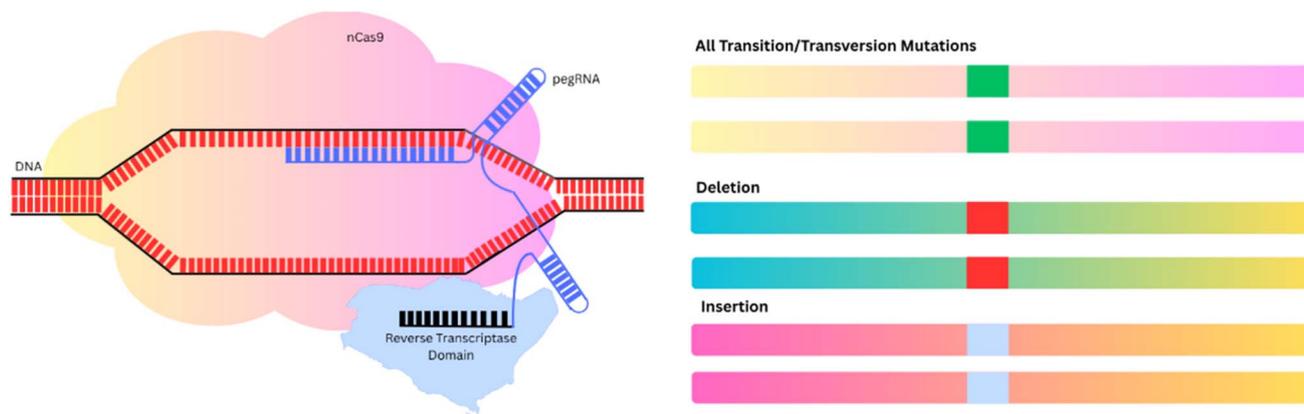


Fig. 6 Prime editing and its derived techniques. Prime editors expand the scope of DNA editing to not all transition and transversion mutations, as well as small insertion and deletion mutations.

Table 1 Comparative overview of genome editing tools used in diverse plant species for trait improvement

Tool	Researcher(s)	Plant species	Target gene(s)/region	Outcome	References
ZFNs	Sylvia de Pater	Arabidopsis	PPO	Herbicide-insensitive enzyme due to gene mutation	70
ZFNs	Vipula K. Shukla	Zea maize	IPK1	Herbicide tolerance; modified inositol phosphate profile	71
TALENs	Qiwei Shan	Brachypodium, rice	Multiple genes	Gene editing <i>via</i> NHEJ; large deletions with dual TALENs	72
TALENs	Yanpeng Wang	Bread wheat	3 homoeoalleles	Powdery mildew resistance	73
TALENs	William Haun	Soybean	FAD2-1A and FAD2-1B	Improved oil quality and shelf life	74
TALENs	Toni Wendt	Barley	Genome-wide	~20% transformation efficiency; small deletions <i>via</i> NHEJ	75
CRISPR	Andrew S. Fister	Cacao	TcNPR3	Resistance to <i>Phytophthora tropicalis</i>	76
CRISPR	Valero Pompili	Apple	MdDIPM4	Potential for improved stress and disease resistance	77
CRISPR	Jun Li	Rice	EPSPS (<i>via</i> NHEJ)	Glyphosate resistance <i>via</i> targeted substitution (TILLING)	78
CRISPR	Anning Zhang	Rice	OsRR22	Enhanced salinity tolerance	79
CRISPR	Xiaohong Sun	Apple (<i>Malus</i> sp.)	MdMCK9	Increased anthocyanin accumulation	80
CRISPR	Akira Endo	Rice	OsOr	Elevated β -carotene levels	81

genetic change. Once the nCas9 makes a single-strand nick near the target site, the RT enzyme uses the pegRNA as a template to synthesize the edited DNA sequence.⁶⁹ This process generates two DNA flaps, an edited 3' flap and an unedited 5' flap. Unedited flap is preferentially degraded by endogenous endonucleases, facilitating incorporation of the edited sequence through cellular repair and replication mechanisms.⁶⁹ The first-generation prime editor (PE1) achieved modest efficiency, which was later improved in PE2 through enhanced RT variants and further optimized in PE3 by adding a second gRNA to nick the opposite DNA strand, boosting the editing efficiency to around 33%.⁶⁹ Prime editing can introduce all twelve types of base substitutions as well as small insertions or deletions, offering higher precision and fewer off-target effects, as shown in Fig. 6. Although its efficiency and specificity are still being refined, prime editing represents a major milestone toward precise, versatile, and potentially therapeutic genome editing.

CRISPR/Cas9 leads to these advancements, providing unmatched precision and efficiency in genome editing. This

integrative approach will enable the development of crops that are not only resistant to diseases, pests, and weeds but also tailored to thrive under specific environmental conditions. Researchers experienced with CRISPR have helped newcomers by openly sharing methods and resources, unlike the restricted, company-controlled framework of zinc-finger nucleases.⁴⁵ Several studies have successfully demonstrated the use of ZFNs, TALENs, and CRISPR/Cas9 in various plant species for precise gene editing to improve agronomic traits. These applications are summarized in Table 1, highlighting the practical outcomes of each technology in enhancing crop resistance, yield, and nutritional quality.

4. Nucleic acid-based diagnostic techniques

Foodborne illnesses pose a significant threat to public health and the global economy. They impact the consumers, food industry, and regulatory systems. Bacterial and viral pathogens are the primary biotic agents compromising food safety.⁸²



Nucleic Acid-Based Detection Techniques

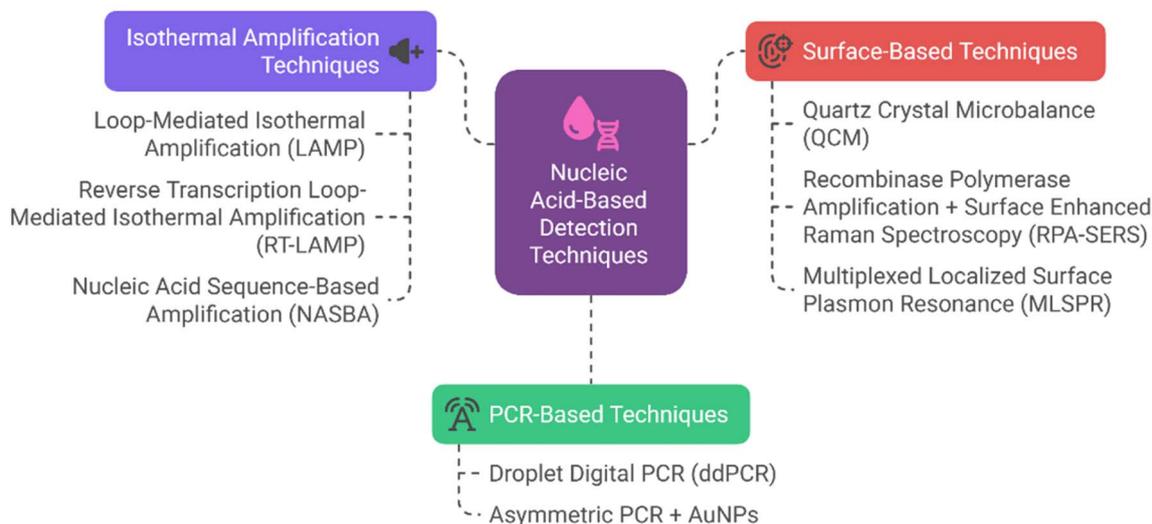


Fig. 7 Types of nucleic acid-based detection techniques.

Traditionally, pathogen detection has relied on culture-based methods, but these are often time-consuming, costly, and labour-intensive. Therefore, nucleic acid-based techniques are widely adopted due to their higher sensitivity, speed and specificity. These molecular tools are increasingly replacing conventional approaches in routine food testing.^{84,85}

Conventional detection methods for plant and foodborne pathogens are broadly categorized as direct and indirect techniques.^{83,85} Direct methods include Polymerase Chain Reaction^{16,86} (PCR), immunoassays,⁸⁷ and culture-based colony counting.⁸⁸ Indirect methods are non-invasive and involve technologies such as thermography, gas chromatography, hyperspectral imaging, and fluorescence imaging.⁸⁹ Despite their accuracy, these standard techniques are often time-consuming, costly, and labour-intensive.⁹⁰ Initially, nucleic acid-based methods were limited to research laboratories due to their complexity and the requirement for skilled personnel.⁹¹ However, with technological advancements these approaches have become more accessible and are replacing culture-based

and immunoassay techniques in routine food safety analysis.^{85,92} Nucleic acid-based technologies also play a critical role in both basic research and applied sciences. They are extensively used in clinical diagnostics, pathogen identification, gene cloning, and industrial quality control.⁸⁵

In recent years, various advanced nucleic acid-based diagnostic methods have emerged that overcome the limitations of conventional assays. These include techniques like Quartz Crystal Microbalance⁹⁰ (QCM) using DNA amplicons, Recombinase Polymerase Amplification Surface-Enhanced Raman Scattering⁹³ (RPA-SERS) and gold nanoparticle-based PCR.⁹⁴ Additionally, isothermal amplification methods such as Loop-Mediated Isothermal Amplification⁹⁵ (LAMP), Reverse Transcription Loop-Mediated Isothermal Amplification⁹⁶ (RT-LAMP), and Nucleic Acid Sequence-Based Amplification⁹⁷ (NASBA) offer rapid detection without the need for thermal cyclers. Multiplexed Localized Surface Plasmon Resonance⁹⁸ (LSPR) approaches allow simultaneous detection of multiple DNA targets with high sensitivity. These technologies are not

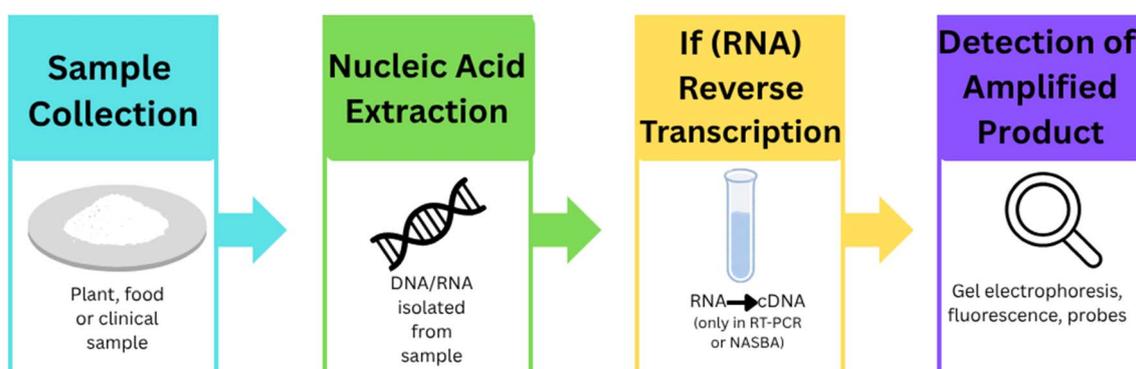


Fig. 8 General workflow of nucleic acid-based detection techniques.



Table 2 Comparison of nucleic acid-based detection techniques

Technique	Target	Amplification type	Isothermal?	Key enzymes/features	Detection method	References
ddPCR	DNA	PCR-based (partitioned)	✗	DNA polymerase; droplet-based quantification	Fluorescence in droplets	103
QCM	DNA	PCR (multiplex/conventional PCR)	✗	PCR-amplified; DNA detected	Mass-change-based acoustic biosensing using QCM	90
RPA-SERS	DNA	Recombinase-based	☑	Recombinase, DNA polymerase, and SSB	Raman signal of labeled probes	93
LAMP	DNA	Polymerase-based loop amplification	☑	DNA polymerase with loop primers	Turbidity, color, and fluorescence	95
RT-LAMP	RNA (converted to cDNA)	Isothermal	☑	Reverse transcriptase + Bst polymerase	Color change, fluorescence, and turbidity	96
NASBA	RNA	Transcription-based	☑	Reverse transcriptase, RNase H, and T7 RNA polymerase	Fluorescence- or probe-based	97
Asymmetric PCR + AuNPs	DNA	PCR (with excess primer)	✗	DNA polymerase; creates more ssDNA	Gold nanoparticle colour shift	94
MISPR	DNA	Not always with amplification	Sometimes	Surface plasmon resonance on nanoparticle arrays	Optical signal based on nanoparticle resonance	104

Table 3 Overview of nucleic acid-based biosensing tools for detecting plant pathogens and genetic markers using integrated amplification and nanotech platforms

Tool/technique	Researcher(s)	Plant species/matrix	Target gene(s)/ pathogen	Outcomes/findings	References
LAMP-QCM	Reona Takabatake	Papaya	Cauliflower mosaic virus 35S promoter and papaya endogenous	Detect genetically modified papaya with high specificity and sensitivity ($\leq 0.05\%$) without requiring PCR instruments	105
RPA-LFD	Y. Zhou	Tomato	Tomato yellow leaf curl virus (TYLCV)	Rapid DNA detection of the TYLCV	106
LAMP	M. K. Prasannakumar	Rice	<i>Sarocladium oryzae</i> and <i>Magnaporthe oryzae</i>	Detection of 100 fg of pathogen DNA	107
RT-PCR	Sunil B. Kokane	Citrus tristiza	<i>Citrus tristeza virus</i> RNA	Real-time RNA detection using RT-PCR with high specificity	108
Multiplex PCR	Nelly Datukishvili	Maize, Soybean	35S, NOS, EPSPS and <i>cry1Ab</i> gene	Identification of new DNA markers in GMO	109
LAMP-QCM	Sirirat Wachiralurpan	Bacterial DNA solution	<i>Listeria monocytogenes</i>	LAMP products monitored by QCM in real-time	110
LAMP-AuNPs (colorimetric)	Mila Djisalov	Mushroom substrate	<i>Trichoderma</i> spp., <i>tefl</i>	AuNP-based colorimetric detection for rapid fungal screening	111

only faster but also suitable for point-of-care and field diagnostics.⁹⁸ A schematic overview of these diagnostic techniques is presented in Fig. 7. Although these techniques differ in their experimental setups, they largely follow a similar core mechanism.⁹⁹ All these methods target and amplify specific nucleic acid sequences (DNA or RNA) of the pathogen to enable sensitive detection. The general mechanism involves four key steps: sample preparation, nucleic acid extraction, amplification, and detection.^{99,100} The process begins with sample collection

followed by nucleic acid extraction (DNA or RNA). If the target is RNA, it is usually converted to complementary DNA (cDNA) using reverse transcription. This is followed by amplification of the target nucleic acid, which can be achieved through isothermal techniques. Finally, detection is carried out using various methods including gel electrophoresis, fluorescence analysis, colorimetric changes, nanoparticle-based sensors (AuNPs), or signal-enhancing platforms such as quartz crystal-based biosensors.^{101,102} A systematic diagram representing this



common mechanism is shown in Fig. 8, while the methodological variations among these techniques are summarized in Table 2. Several studies have successfully demonstrated the use of LAMP,⁹⁵ QCM,⁹⁰ and NASBA⁹⁷ in various plant species for detection of plant pathogens to improve agronomic traits. These applications summarized in Table 3, demonstrate the use of nucleic acid-based biosensing tools for sensitive and rapid detection of plant pathogens and genetic markers using integrated amplification and nanotechnology platforms. Several other technologies are also used to detect plant pathogens such as CRISPR-based diagnostics¹⁰¹ that represent a transformative approach in the field of molecular diagnostics, offering rapid, accurate, and sensitive detection capabilities across various applications. These diagnostics leverage the CRISPR/Cas system, originally known for its gene-editing capabilities, to identify specific nucleic acid sequences, making them highly versatile tools for detecting pathogens, genetic mutations, and invasive species. While CRISPR-based diagnostics offer significant advantages, challenges remain in terms of scalability and integration into existing diagnostic frameworks. Future developments may focus on automation and modularization to enhance the accessibility and efficiency of these diagnostics in various settings.¹⁰²

5. RNA-based gene silencing

Sustainable production strategies including the development of safer pesticide alternatives are critically required to improve the current cropping system. The RNA interference (RNAi) mechanism has become a promising approach for effectively managing phytophagous pests and combating pathogenic attacks.¹¹² With its inherent capability for the sequence-specific target, this technology is rapidly becoming a major focus of research as an environmentally sustainable and cost-efficient solution for pest management.¹¹³ It is a conserved eukaryotic mechanism that regulates gene expression at either the post-transcriptional level (post-transcriptional gene silencing (PTGS)) or the transcriptional level (transcriptional gene silencing (TGS)).¹¹⁴ TGS halts transcription by methylating the 5'-untranslated region (5' UTR), preventing the binding of transcription factors, while in PTGS methylation of the coding region marks the transcript for degradation.¹¹⁵ In fungi, this PTGS mechanism is referred to as quelling.^{116,117}

In agriculture, RNAi has been widely utilized, especially for developing resistance to biotic stressors, bacteria, nematodes, fungi, and viruses.¹¹⁸ This phenomenon was initially discovered in the free-living nematode *Caenorhabditis elegans*.¹¹⁹ The RNAi mechanism is used to inhibit key growth and developmental genes in targeted phytophagous pests at the post-transcriptional level that helps to mitigate their harmful impact on crop plants. RNAi is activated by double strand RNA (dsRNA) and multidomain enzymes, such as the RNase III classes of enzymes in the Dicer family, which convert dsRNA into small interference RNA (siRNA), a double-strand RNA.^{120,121} These siRNAs are fused with the RNA induced silencing complex (RISC) having Argonaute (AGO) proteins. This RISC

complex and siRNA complement with viral RNA and cleave/degrade the viral RNA¹²² as shown in Fig. 9.

There are two sources of dsRNA *i.e.* endogenous dsRNA (inside the cell) and exogenous dsRNA (outside the cell).^{123,124} The introduction of exogenous dsRNA in plants involves various approaches, depending on the specific research objectives and intended applications. These approaches have been widely investigated in plants to examine gene functions *via* reverse genetics and to attain crop enhancement objectives through genetic modifications.¹¹⁵ The major strategies of dsRNA introduction in plants to protect from pests are Host Induced Gene Silencing¹²⁵ (HIGS), Spray Induced Gene Silencing¹²⁵ (SIGS), and a specialized RNAi-based approach – Virus Induced Gene Silencing¹²⁶ (VIGS).

In HIGS, transgene plants (wheat, barley, rice, and maize) are genetically modified to produce dsRNA, which is taken by pests to silence the insect critical gene and protect plants from degradation¹²⁵ as shown in Fig. 10a.

In SIGS, dsRNA is synthesized *in vitro* which targets the essential gene in pathogens. Synthesized dsRNA is sprayed onto the plant surface (strawberry, lettuce, canola, and barley) by using nanocarriers or protective formulations which are absorbed by the pest or pathogen, either through feeding or direct contact. As a result, silencing of the targeted gene takes place inside the target organism¹²⁵ as shown in Fig. 10b.

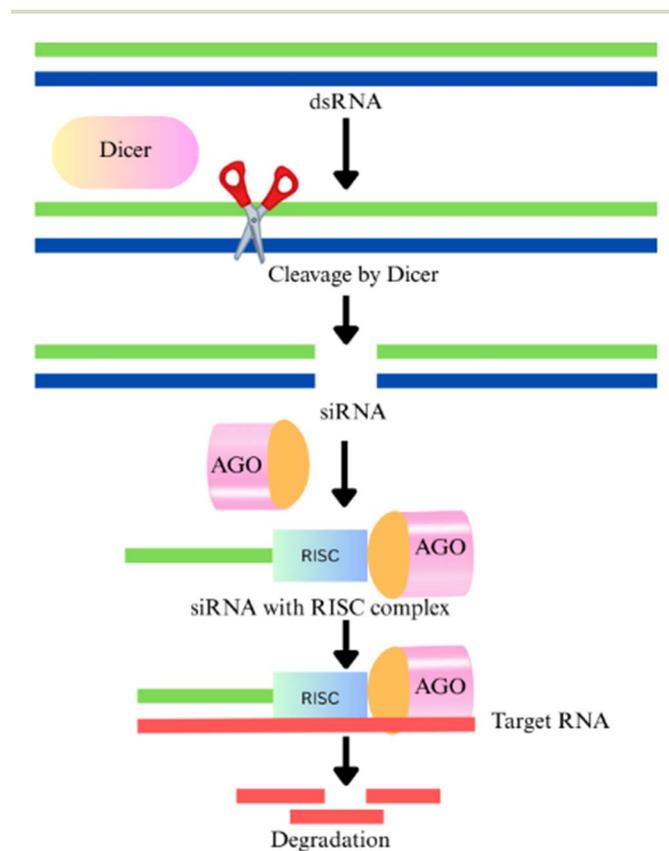


Fig. 9 Mechanism of RNA silencing incorporating the Dicer family enzyme, RNA-induced silencing complex (RISC) formation and degradation of viral RNA.



Virus induced gene silencing is a specialized RNAi-based approach that uses engineered plant viruses to assess gene functions in plants and control plant pests. Once the virus infects the plant, it triggers the plant defense mechanism. The plant produces dsRNA as a part of its response to the viral infection, and as a result silencing of gene takes place¹²⁶ as shown in Fig. 10c. It offers significant advantages for functional genomics as it is rapid, does not require stable transformation, and also allows high-throughput screening of candidate genes involved in stress responses, defense mechanisms, and developmental processes. This makes VIGS particularly valuable for studying gene function in non-model plants or those with long generation times. Xijun Chen¹²⁷ explored the use of composite nanomaterials as delivery systems for dsRNA to enhance plant protection against viral pathogens.

Artificial dsRNA produces a large population of siRNAs, out of which only a limited subset is functionally active in guiding RISC mediated target cleavage. The remaining, non-functional siRNAs can contribute to off-target effects, thereby reducing the overall efficiency and specificity of RNA silencing.¹²⁸ However, a major challenge with topical application of naked

dsRNA is its instability on the plant surface, leading to limited protection duration. Neena Mitter¹²⁹ and her colleagues developed a novel nanocarrier system called BioClay, which uses Layered Double Hydroxide (LDH) nanosheets to deliver dsRNA in a stable form. When dsRNA is loaded onto these clay nanosheets, it becomes protected from environmental degradation, including nucleases and rain. Similarly, Marie Knoblich¹³⁰ and colleagues developed an experimental platform termed 'eNA screen', which utilizes lysates from cultured *Nicotiana tabacum* BY-2 cells (BY-2 lysates). This system enables the identification of 'effective siRNAs' (esiRNAs) which are capable of directing efficient AGO/RISC-mediated target RNA cleavage. The application of the eNA screen to cucumber mosaic virus (CMV) allowed the identification of esiRNAs with strong antiviral activity, demonstrating their potential in conferring resistance against CMV infection.

RNAi can be effectively used to enhance the yield of crops, fruits and plants by modifying key agronomic traits such as plant height, branching, and size. Kenneth A. Feldmann¹³¹ employed RNAi to reduce the activity of the gene *OsDWARF4* of rice that resulted in shorter plants with their leaves growing

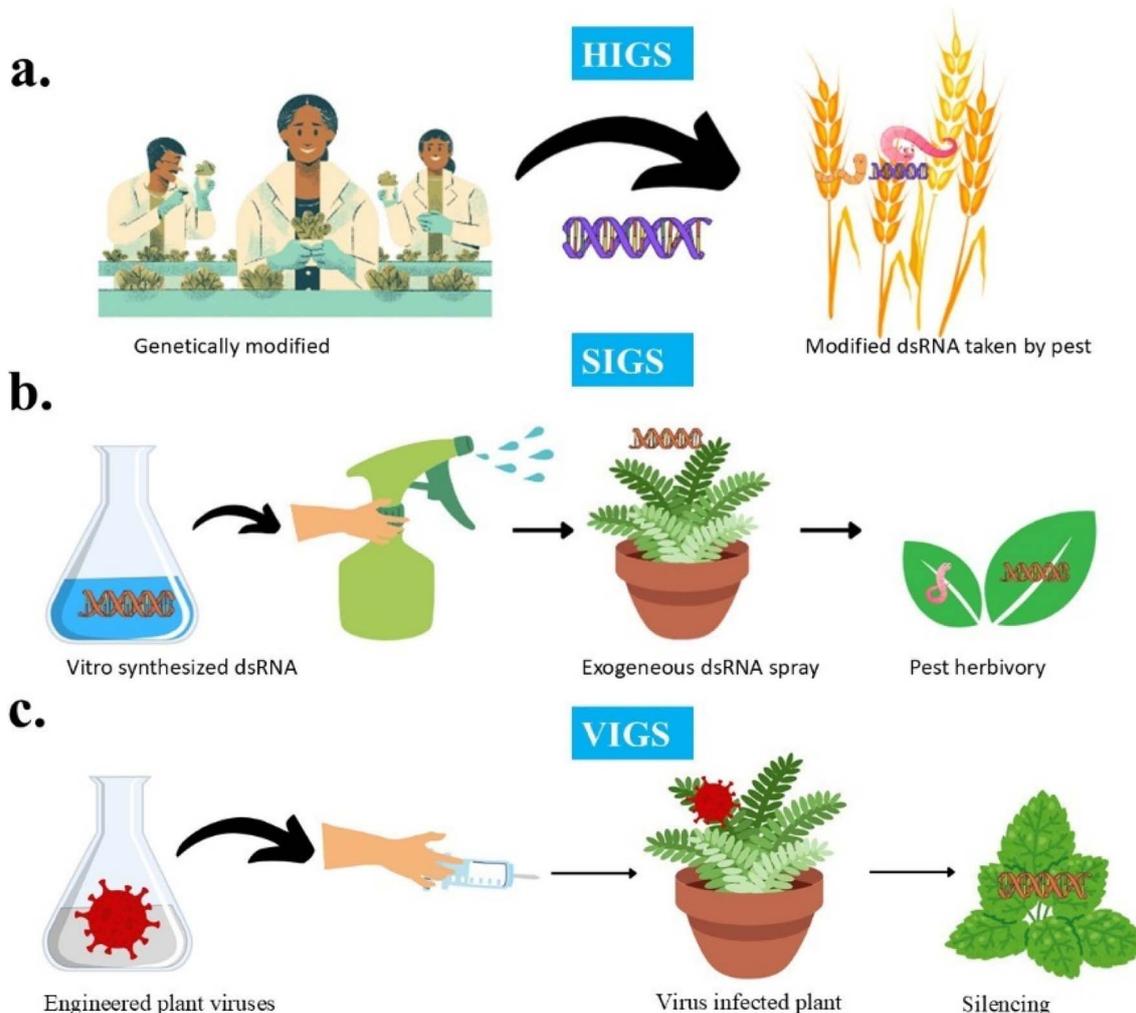


Fig. 10 Strategies for dsRNA delivery for pest/pathogen control. (a) Host Induced Gene Silencing (HIGS), (b) Spray Induced Gene Silencing (SIGS) and (c) Virus Induced Gene Silencing (VIGS).



more upright instead of drooping, thus this increases light interception, leading to higher photosynthesis, and improved yields are observed under dense planting conditions. Hisano *et al.*¹³² used RNAi in the downregulation of some lignin genes like cinnamate 4-hydroxylase (C4H), shikimate hydroxycinnamoyl transferase (HCT) and 4-coumarate:CoA ligase (4CL) in plants that resulted in the reduction of lignin content, improved accessibility of cellulases for cellulose degradation, and increased dry matter degradability. Qiao *et al.*¹³³ employed RNAi to reduce the activity of the enzyme GA 20-oxidase (OsGA20ox2) that resulted in a decrease in the length of rice variety named QX1, which helps in increasing the panicle length, number of seeds per panicle and test weight (1000 grains). Jiao¹³⁴ and Miura¹³⁵ observed that the OsSPL16 gene is a positive regulator of cell proliferation with an increase in grain width and yield in over-expressing rice plants. Its over-expression decreases the grain appearance and quality therefore, Jiao¹³⁴ and Miura¹³⁵ decreased the expression of OsSPL16 gene and resulted in slender grains with better quality. Davuluri *et al.*¹³⁶ suppressed the DET1 gene in tomato that resulted in an increase in the level of flavonoid and carotenoid, which are highly beneficial for human health. Yu *et al.*¹³⁷ suppressed the expression of lycopene epsilon cyclase (ϵ -CYC) gene by using RNAi and resulted in the enhancement of carotenoid content of rapeseed (*Brassica napus*). Dandekar *et al.*¹³⁸ also utilized RNAi

in apple to improve the fruit quality by enhanced self-life. Regina *et al.*¹³⁹ used RNAi to down-regulate the starch-branching enzyme resulting in high-amylose wheat, which has great potential to improve human health. Gil Humanes *et al.*¹⁴⁰ silenced the expression of specific γ -gliadins in different wheat cultivars and resulted in the enhancement of protein content of transgenic lines.

6. Integration of artificial intelligence with genome editing techniques for enhanced precision

Artificial Intelligence (AI) is being integrated into genome-editing pipelines to overcome major bottlenecks in precision and predictability. Deep learning and machine learning (ML) models are widely used to design tools with high efficiency, generate efficient gRNAs with minimal off-target effects by analyzing sequence performance patterns from extensive data. AI platforms such as DeepCRISPR,¹⁴¹ ZFN-Site,¹⁴³ CCTop¹⁴³ and siRNA-Finder¹⁴⁴ enable prediction of off-target sites with far greater accuracy than rule-based tools. In base and prime editing ML frameworks help in optimizing the sequence context, thereby reducing byproducts and improving editing fidelity. AI-driven prediction supports the selection of

Table 4 Computational tools and webserver for different nucleic acid-based techniques

Software/Tool	Purpose	References
PrimerExplorer	A widely used software for designing LAMP primers, offering a user-friendly interface for constructing primers for loop-mediated isothermal amplification	146
NEB LAMP primer design tool	A tool provided by New England Biolabs for designing LAMP primers, gaining popularity for its ease of use and specificity	146
FastPCR	An integrated tool for designing primers for various PCR applications, including LAMP, multiplex PCR, and long-distance PCR	147
ThermoPlex	An automated design tool for target-specific multiplex PCR primers based on DNA thermodynamics, ensuring optimal primer design	149
PrimerJinn	A tool for designing multiplex PCR primer sets and performing <i>in silico</i> PCR evaluation, particularly for targeted sequencing of pathogens	150
PMPrimer	A Python-based tool for automated design and evaluation of multiplex PCR primer pairs using diverse templates	151
PrimerScore2	A high-throughput primer design tool that uses a piecewise logistic model to score primers for multiple PCR variants	152
SADDLE	A stochastic algorithm for designing highly multiplex PCR primer sets with minimal primer dimer formation	153
MOPSO-based primer design	A multiobjective particle swarm optimization (MOPSO) approach for designing primers based on user-specified parameters	154
Ultiplex	A web-based multiplex PCR primer design tool that supports up to 100-plex multiplicity and includes compatibility checking for primer groups	155
NGS-PrimerPlex	A command-line application for designing primers for amplicon-based genome target enrichment in multiplex PCR	156
MRPrimerW	A web-based tool for designing high-quality primers for multiple target qPCR experiments, including homology testing and TaqMan probes	157
GPrimer	A GPU-based pipeline for rapid primer design, significantly improving computational speed compared to traditional methods	158
PrimerServer	A high-throughput primer design and specificity-checking platform with web and command-line interfaces for large-scale applications	159



Table 5 Databases used to provide target sequences for different techniques

Database	Purpose	References
EENdb	Database for ZFNs and TALENs, providing target sequences and efficiency data	160
PICKLES	Provides data from pooled CRISPR knockout screens	161
Cas-database	Designs genome-wide guide RNA libraries for CRISPR screens	162
CHOPCHOP	Designs CRISPR/Cas9 and TALEN constructs	22
grID	Guides RNA database with gRNA properties	163
CRISPRz	Validates CRISPR target sites in zebrafish	164
CRISPRdirect	Designs target site-specific gRNA sequences	165

biologically relevant targets before experimental validation. In plants, AI also assists in optimizing delivery constructs, regulatory elements, and tissue specific expression strategies. Collectively, the integration of AI with genome editing substantially enhances edit precision, reduces experimental iterations, and accelerates the development of reliable, high-performance, crop-improvement strategies.

7. Computational resources for precision genome editing and nucleic acid-based biosensing

AI models can analyze large genomic datasets to optimize gRNA selection, predict off-target effects, and model protein–DNA/RNA interactions with high accuracy. Platforms such as DeepCRISPR¹⁴¹ and CNN-based gRNA scoring¹⁴⁵ systems exemplify how AI is transforming genome engineering into a more predictive, efficient, and scalable process. Over the past few years, various computational technologies utilizing AI and ML have been designed for target identification, predicting/reducing off-target activity of gene editing and gene regulation methodologies. In addition to genome editing, AI/ML approaches are also being employed in nucleic acid-based detection techniques such as droplet digital PCR¹⁰³ (ddPCR) and LAMP¹⁰⁷ where they assist in designing specific primers and probes, enhance signal analysis from biosensor platforms and improve multiplex detection efficiency. Several dedicated tools are now available for designing primers, like PrimerExplorer,¹⁴⁶ NEB LAMP Primer¹⁴⁶ Design Tool, FastPCR,¹⁴⁷ *etc.*, as listed in Table 4. Databases used for providing target sequences in ZFN, TALEN, CRISPR/Cas9 and RNAi are presented in Table 5. Off-target activity encompasses mainly three major categories: software tools and packages, specialized databases, and web-based platforms, each of which exhibits diverse areas of application. Certain resources are tailored for specific genomes, whereas others offer broader compatibility, accommodating any user-defined genomic input. While some tools prioritize computational speed and scalability for larger genomes, others are optimized for precise algorithmic control and customization.¹⁴⁸ Common features shown by each tool are off-targets, scoring, ranking and genome specificity. Computational tools and their detailed comparison of off-target feature are presented in Table 6.

8. Conclusions and future prospects

Gene editing and nucleic acid-based technologies are opening new possibilities in plant biotechnology. These tools offer accurate and sustainable methods to boost crop yield, improve disease resistance, and ensure food security. Among them, genome editing tools like ZFNs, TALENs, and CRISPR/Cas9 have transformed plant breeding. They allow targeted changes in the plant genome by creating DSBs, which are repaired by the plant's natural mechanisms. On the other hand, the base editing and prime editing tools enable precise, programmable nucleotide changes without DSBs. While ZFNs and TALENs provided the early framework for gene editing, CRISPR/Cas9 is now most widely used. Its popularity comes from its simplicity, high precision, and ease of design. The capacity to induce targeted genetic modifications enables the precise introduction or alteration of desirable traits in elite crop varieties utilized for breeding and agricultural production. Moreover, the potential to stack multiple genetic modifications offers opportunities for the development of multi-trait resistance and the controlled expression of pharmaceutically important proteins. However, challenges such as off-target mutations, low editing accuracy, and inefficient delivery methods still need to be addressed. While no system has yet been established as most efficient and safe, ongoing efforts, particularly computational approaches aimed at minimizing off-target effects, hold promise for enhancing the precision and overall efficacy of therapeutic applications.

Alongside gene editing, nucleic acid-based diagnostic methods are essential for plant health monitoring. Techniques such as PCR¹⁶ and LAMP⁹⁵ offer fast and sensitive detection of pathogens, even at early stages. These diagnostics are being improved through integration with nanotechnology. These enhancements make them suitable for field-level use and point-of-care testing. This helps in reducing yield loss caused by undetected plant diseases.

In recent years, the global scientific and regulatory communities have been engaged in an ongoing debate regarding whether the application of these genome editing techniques falls within the framework of existing genetically modified organism legislation.¹⁹⁹ Regulatory clarity for New Genomic Techniques (NGTs) is still evolving. The European Food Safety Authority (EFSA) concludes that cisgenic and intragenic plants developed through NGTs do not pose new or additional risks compared to conventionally bred plants and



Table 6 Computational tools, database and webserver for different genome editing techniques

ZFN-based			
ZFN tools	Type	Off-targets	References
Zinc Finger tools	Tool	✗	166
ZifBASE	Database	✗	167
ZiFiT (v4.2)	Tool	✓	168
ZFN-site	Tool	✓	142
TALEN-based			
TALEN tools	Type	Off-targets	References
TALE-NT 2.0	Tool	✓	169
TALENoffer	Package	✓	170
E-TALEN	Tool	✓	171
SAPTA	Tool	✓	172
CRISPR-based			
CRISPR tools	Type	Off-targets	References
Cas9 design	Tool and database	✓	173
CasOT	Tool	✓	174
Cas-OFFinder	Tool	✓	175
CRISPR optimal target finder	Tool	✓	176
E-CRISP	Tool	✓	177
CRISPR-P	Tool	✓	178
GT-SCAN	Tool	✓	179
CRISPy	Tool	✓	180
sgRNAcas9	Package	✓	181
CRISPRseek	Package	✓	182
COSMID	Tool	✓	21
CRISPRdirect	Tool	✓	183
Off-Spotter	Tool	✓	184
CRISPR multitargeter	Tool	✗	185
CCTop	Tool	✓	143
CrisprGE	Database	✓	186
WGE	Package	✓	187
ZFN/TALEN/CRISPR-Cas-based			
Tools	Type	Off-targets	References
EENdb	Database	✗	160
Mojo hand	Tool	✓	188
CHOPCHOP	Tool	✓	22
PROGNOS	Tool	✓	189
RNAi-based			
RNAi tools	Type	Applications	References
pssRNAit	Tool	Designing siRNAs	190
siRNA-Finder	Tool	Designing siRNAs	144
sPARTA	Tool	Analyzing miRNA	191
AttSiOff	Tool	Off-targets	192
MIRZA-G	Tool	Off-targets	193
siRNADesign	Tool	Off-targets	194
dsRNAEngineer	Tool	Designing dsRNAs	195
kmerPMTF	Tool	Predicting miRNA	196
PAREameters	Tool	Identifying miRNA	197
RNA Degradome	Tool	Predicting sRNA	198

recommends risk-proportionate, science-based assessment criteria.²⁰⁰ The US Department of Agriculture decides in a case-by-case manner and has stated on requests that small mutations in corn induced by ZFNs fall outside their scope of regulation.²⁰¹

RNA interference (RNAi) technologies, including siRNA and miRNA, have become valuable for plant researchers. These techniques are employed not only to explore plant functions but also to engineer plants with enhanced or novel traits through the manipulation of both beneficial and detrimental genes. This technology has been effectively applied in crops to enhance not only food productivity (such as biomass and grain yield) but also their nutritional value, with cereals, fruits, and vegetables being enriched with essential minerals, vitamins, fatty acids, and amino acids. RNAi technology has also been exploited to develop plants with improved resistance against various environmental stresses (especially drought). Although RNAi technology can serve as a potential tool for crop improvement, certain limitations are also associated with it. Altering the expression of a target gene might lead to undesirable changes in plant morphology and development; therefore, transgenic strategies should be designed only after completely understanding the mechanism of its regulation. siRNA-based RNAi strategies might not be suitable for some applications requiring tissue-specific silencing of genes.²⁰²

In coming years, ongoing advancements and innovations are expected to yield more robust computational frameworks for genome editing. Computational tools and AI play key roles in advancing these technologies. They help in improving the accuracy, speed, and scalability of genome editing, diagnostics, and RNAi technologies. Tools including grID,¹⁶³ CHOPCHOP,²² PrimerExplorer,¹⁴⁶ siFi¹⁴⁴ and sPARTA¹⁷² assist in designing gRNAs, primers, and siRNAs. These tools reduce off-target effects and improve the targeting efficiency. Specialized databases also support gene selection, off-target prediction, and RNA structure analysis. Together, they make molecular tools more reliable and user-friendly. Notably, recent computational studies demonstrated the potential of modified siRNA molecules to effectively inhibit viral RNA-silencing suppressor proteins, such as p19, thereby restoring the RNAi mechanism and improving resistance against plant viruses.²⁰³ These emerging tools and algorithms hold the potential to significantly enhance genome editing and nucleic acid-based technologies by contributing to both pre-editing design and post-editing analysis, thereby expanding their applicability.

Looking ahead, combining genome editing, RNA-based methods, diagnostics, and AI could transform agriculture. These integrated systems will help develop crops that are more resilient, high-yielding, and rich in nutrients. These approaches will also allow precision breeding tailored to the local environment and nutritional needs. The integration of genome editing with synthetic biology could pave the way for designing new metabolic pathways and creating biofortified crops to tackle malnutrition. However, the widespread application of these technologies will also require robust regulatory frameworks, ethical considerations, and increased public awareness to ensure their safe and acceptable use. Promoting farmer



education and consumer acceptance will be essential for realizing the full potential of nucleic acid research in ensuring sustainable food security for future generations. In future, tools that exhibit substantial potential in terms of their applicability may consequently be envisioned as among the most widely adopted next-generation technologies for both scientific and therapeutic applications.

In conclusion, multiple proof-of-concept studies have already demonstrated the practical success of various technologies. CRISPR-edited rice lines with OsRR22 mutations show enhanced salinity tolerance,⁷⁹ TALEN-mediated *MLO* knockouts in wheat confer powdery mildew resistance⁷³ and ZFN-engineered IPK1 maize exhibits herbicide tolerance.⁷¹ Likewise, LAMP-QCM assays detect GMO papaya with 0.05% sensitivity.¹⁰² Such validated examples collectively confirm that nucleic-acid-based editing, diagnostics, and RNAi technologies are not merely theoretical innovations but proven tools with strong translational potential in agriculture. They offer precise, efficient, and eco-friendly solutions to address global challenges such as food insecurity and climate change. Continued innovation, supportive policies, and responsible use will ensure these technologies become foundational for resilient food systems in the future.

9. Limitations and biosafety considerations

Notwithstanding the accelerating developments in genome editing and RNA-based methodologies for enhancing crop traits, a number of considerable challenges remain unresolved. The efficacy of introducing editing tools is severely compromised in plant species characterized by complex genomic architectures or strong inherent biological barriers. Off-target effects and variable editing efficiency restrict the reproducibility and stability of edited traits. The stability and successful cellular uptake of dsRNA in RNAi-based applications are highly susceptible to ambient environmental parameters.

From a biosafety standpoint, the comprehensive assessment of ecological and regulatory implications is imperative. Elevated risks require analysis, specifically concerning unintended genetic dispersal (gene flow), non-specific gene silencing in organisms outside the target scope, and the environmental persistence of the introduced nucleic acids or editing machinery. Risk assessment frameworks and public acceptance also play critical roles in translating laboratory advances into sustainable agricultural solutions.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

New data are not generated as a part of the submitted study.

Acknowledgements

Yashika acknowledges Netaji Subhas University of Technology for funding.

References

- 1 A. Faizal, S. Nugroho, A. A. Sembada, Y. Theda, T. Komariyah and R. R. Esyanti, Genome Editing in Future Crop Protection: Utilizing CRISPR/Cas9 to Improve Crop Resistance against Diseases, Pests, and Weeds, *Discov. Agric.*, 2024, **2**(1), 104.
- 2 S. Fiaz, S. Ahmar, S. Saeed, A. Riaz, F. Mora-Poblete and K. H. Jung, Evolution and application of genome editing techniques for achieving food and nutritional security, *Int. J. Mol. Sci.*, 2021, **22**(11), 5585.
- 3 S. He and K. M. Creasey Krainer, Pandemics of People and Plants: Which Is the Greater Threat to Food Security?, *Mol. Plant*, 2020, **13**(7), 933–934.
- 4 Food and Agriculture Organization (FAO) of the United Nations, *Climate Change Fans Spread of Pests and Threatens Plants and Crops, New FAO Study*. Rome, 2021.
- 5 R. N. Strange and P. R. Scott, Plant Disease: A Threat to Global Food Security, *Annu. Rev. Phytopathol.*, 2005, **43**, 83–116.
- 6 UNDESA. *World Population Prospects, the 2022 Revision; Population Division*, Department of Economic and Social Affairs, United Nations, New York, NY, USA, 2022.
- 7 E. K. Norrman, World Population Growth: A Once and Future Global Concern, *World*, 2023, **4**, 684–697.
- 8 S. K. Patel, A. Sharma and G. S. Singh, Traditional agricultural practices in India: an approach for environmental sustainability and food security, *Energy, Ecol. Environ.*, 2020, **5**(4), 253–271.
- 9 A. O. Adefila, O. O. Ajayi, A. S. Toromade and N. J. Sambulya, Integrating traditional knowledge with modern agricultural practices: A sociocultural framework for sustainable development, *World J. Biol. Pharm. Health Sci.*, 2024, **20**(02), 125–135.
- 10 T. Johns, B. Powell, P. Maundu and P. B. Eyzaguirre, Agricultural biodiversity as a link between traditional food systems and contemporary development, social integrity and ecological health, *J. Sci. Food Agric.*, 2013, **93**(14), 3433–3442.
- 11 H. N. Barman, Z. Sheng, S. Fiaz, M. Zhong, Y. Wu, Y. Cai, W. Wang, G. Jiao, S. Tang, X. Wei and P. Hu, Generation of a New Thermo-Sensitive Genic Male Sterile Rice Line by Targeted Mutagenesis of TMS5 Gene through CRISPR/Cas9 System, *BMC Plant Biol.*, 2019, **19**(1), 109.
- 12 R. Manshardt *Crop Improvement by Conventional Breeding or Genetic Engineering: How Different Are They*, 2004. <http://www.ctahr.hawaii.edu>.
- 13 K. P. Voss-Fels, A. Stahl, B. Wittkop, C. Lichthardt, S. Nagler, T. Rose, T. W. Chen, H. Zetzsche, S. Seddig, M. Majid Baig, A. Ballvora, M. Frisch, E. Ross, B. J. Hayes, M. J. Hayden, F. Ordon, J. Leon, H. Kage, W. Friedt, H. Stützel and R. J. Snowdon, Breeding Improves Wheat



- Productivity under Contrasting Agrochemical Input Levels, *Nat. Plants*, 2019, 5(7), 706–714.
- 14 M. C. Lallawmkimi, P. Ashoka, D. J. M. S. N. K. S. Veda, A. Yadav, B. Dhivya, M. Kumar and A. Rout, Innovative Approaches in Crop Genetic Engineering for Sustainable Agriculture: A Review, *J. Adv. Biol. Biotechnol.*, 2024, 27(8), 615–631.
- 15 S. Fiaz, S. Ahmar, S. Saeed, A. Riaz, F. Mora-Poblete and K. H. Jung, Evolution and Application of Genome Editing Techniques for Achieving Food and Nutritional Security, *Int. J. Mol. Sci.*, 2021, 22(11), 5585.
- 16 M. Jalali, J. Zaborowska and M. Jalali, The Polymerase Chain Reaction: PCR, QPCR, and RT-PCR, in *Basic Science Methods for Clinical Researchers*, 2017, pp. 1–18.
- 17 S. Lei, S. Chen and Q. Zhong, Digital PCR for Accurate Quantification of Pathogens: Principles, Applications, Challenges and Future Prospects, *Int. J. Biol. Macromol.*, 2021, 184, 750–759.
- 18 C. T. Scott, The zinc finger nuclease monopoly, *Nat. Biotechnol.*, 2005, 23(8), 915–919.
- 19 K. Chen and C. Gao, TALENs: Customizable Molecular DNA Scissors for Genome Engineering of Plants, *J. Genet. Genomics*, 2013, 40(6), 271–279.
- 20 S. Mishra, S. Nayak, N. Tuteja, S. Poosapati, D. M. Swain, R. K. Sahoo, CRISPR/Cas Mediated Genome Engineering in Plants: Application and Future Prospective, preprint, 2024, DOI: [10.20944/preprints202404.0435.v2](https://doi.org/10.20944/preprints202404.0435.v2).
- 21 T. J. Cradick, P. Qiu, C. M. Lee, E. J. Fine and G. Bao, COSMID: A Web-Based Tool for Identifying and Validating CRISPR/Cas off-Target Sites, *Mol. Ther.–Nucleic Acids*, 2014, 3(12), e214.
- 22 T. G. Montague, J. M. Cruz, J. A. Gagnon, G. M. Church and E. Valen, CHOPCHOP: A CRISPR/Cas9 and TALEN Web Tool for Genome Editing, *Nucleic Acids Res.*, 2014, 42(W1), W401–W407.
- 23 M. K. Samanta, A. Dey and S. Gayen, CRISPR/Cas9: An Advanced Tool for Editing Plant Genomes, *Transgenic Res.*, 2016, 25, 561–573.
- 24 F. D. Urnov, E. J. Rebar, M. C. Holmes, H. S. Zhang and P. D. Gregory, Genome Editing with Engineered Zinc Finger Nucleases, *Nat. Rev. Genet.*, 2010, 11, 636–646.
- 25 A. Rasheed, R. A. Gill, M. U. Hassan, A. Mahmood, S. Qari, Q. U. Zaman, M. Ilyas, M. Aamer, M. Batool, H. Li and Z. Wu, A Critical Review: Recent Advancements in the Use of Crispr/Cas9 Technology to Enhance Crops and Alleviate Global Food Crises, *Curr. Issues Mol. Biol.*, 2021, 43(3), 1950–1976.
- 26 C. Wyman and R. Kanaar, DNA Double-Strand Break Repair: All's Well That Ends Well, *Annu. Rev. Genet.*, 2006, 40, 363–383.
- 27 T. Gaj, C. A. Gersbach and C. F. Barbas, ZFN, TALEN, and CRISPR/Cas-Based Methods for Genome Engineering, *Trends Biotechnol.*, 2013, 31(7), 397–405.
- 28 S. A. Wolfe, L. Nekudova and C. O. Pabo, DNA Recognition by Cys 2 His 2 Zinc Finger Proteins, *Annu. Rev. Biophys.*, 2000, 29, 183–212.
- 29 É. S. Vanamee, S. Santagata and A. K. Aggarwal, FokI Requires Two Specific DNA Sites for Cleavage, *J. Mol. Biol.*, 2001, 309(1), 69–78.
- 30 J. Bitinaite, D. A. Wah, A. K. Aggarwal and I. Schildkraut, FokI Dimerization Is Required for DNA Cleavage, *Biophys. Comput. Biol.*, 1998, 95(18), 10570–10575.
- 31 D. Carroll, Genome Engineering with Zinc-Finger Nucleases, *Genetics*, 2011, 188(4), 773–782.
- 32 A. A. Nemudryi, K. R. Valetdinova, S. P. Medvedev and S. M. Zakian, TALEN and CRISPR/Cas Genome Editing Systems, *Tools Discovery*, 2014, 6, 19–40.
- 33 Editorial, Method of the Year 2011, *Nat. Methods*, 2012, 1, DOI: [10.1038/nmeth.1852](https://doi.org/10.1038/nmeth.1852).
- 34 S. Schornack, A. Meyer, P. Römer, T. Jordan and T. Lahaye, Gene-for-Gene-Mediated Recognition of Nuclear-Targeted AvrBs3-like Bacterial Effector Proteins, *J. Plant Physiol.*, 2006, 163(3), 256–272.
- 35 A. J. Bogdanove and D. F. Voytas, TAL Effectors: Customizable Proteins for DNA Targeting, *Science*, 2011, 333, 1843–1846.
- 36 C. Bassi, J. Ho, T. Srikumar, R. J. O. Dowling, C. Gorrini, S. J. Miller, T. W. Mak, B. G. Neel, B. Raught and V. Stambolic, Nuclear PTEN Controls DNA Repair and Sensitivity to Genotoxic Stress, *Science*, 2013, 341(6144), 395–399.
- 37 L. W. Seymour and A. J. Thrasher, Gene Therapy Matures in the Clinic, *Nat. Biotechnol.*, 2012, 30, 588–593.
- 38 A. Knoll, F. Fauser and H. Puchta, DNA Recombination in Somatic Plant Cells: Mechanisms and Evolutionary Consequences, *Chromosome Res.*, 2014, 22, 191–201.
- 39 A. Bhardwaj and V. Nain, TALENs—an Indispensable Tool in the Era of CRISPR: A Mini Review, *J. Genet. Eng. Biotechnol.*, 2021, 19(1), 125.
- 40 Y. Ishino, H. Shinagawa, K. Makino, M. Amemura and A. Nakata, Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product, *J. Bacteriol.*, 1987, 169(12), 5429–5433.
- 41 R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, A. D. Romero and P. Horvath, CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes, *Science*, 2007, 315(5819), 1709–1712.
- 42 S. Rauf, A. Shahid, M. Faizan, M. N. Khalid and I. Amjad, CRISPR/CAS: A potential tool for genomes editing, *Agrobiol. Rec.*, 2024, 15, 13–23.
- 43 F. Akram, S. Sahreen, F. Aamir, I. u. Haq, K. Malik, M. Imtiaz, W. Naseem, N. Nasir and H. M. Waheed, An Insight into Modern Targeted Genome-Editing Technologies with a Special Focus on CRISPR/Cas9 and Its Applications, *Mol. Biotechnol.*, 2023, 65, 227–242.
- 44 M. Lyu, Y. Sun, N. Yan, Q. Chen, X. Wang, Z. Wei, Z. Zhang and K. Xu, Efficient CRISPR/Cas9-Mediated Gene Editing in Mammalian Cells by the Novel Selectable Traffic Light Reporters, *Int. J. Biol. Macromol.*, 2023, 243, 124926.
- 45 S. Fiaz, S. Ahmad, M. A. Noor, X. Wang, A. Younas, A. Riaz, A. Riaz and F. Ali, Applications of the CRISPR/Cas9 system



- for rice grain quality improvement: perspectives and opportunities, *Int. J. Mol. Sci.*, 2019, **20**(4), 888.
- 46 K. S. Makarova, Y. I. Wolf, J. Iranzo, S. A. Shmakov, O. S. Alkhnbashi, S. J. J. Brouns, E. Charpentier, D. Cheng, D. H. Haft, P. Horvath, S. Moineau, F. J. M. Mojica, D. Scott, S. A. Shah, V. Siksnys, M. P. Terns, Č. Venclovas, M. F. White, A. F. Yakunin, W. Yan, F. Zhang, R. A. Garrett, R. Backofen, J. van der Oost, R. Barrangou and E. V. Koonin, Evolutionary Classification of CRISPR-Cas Systems: A Burst of Class 2 and Derived Variants, *Nat. Rev. Microbiol.*, 2020, **18**, 67–83.
- 47 Y. Xie, S. I. U. Haq, X. Jiang, D. Zheng, N. Feng, W. Wang and Q. S. Qiu, Plant genome editing: CRISPR, base editing, prime editing, and beyond, *Grassl. Res.*, 2022, **1**(4), 234–243.
- 48 N. M. Gaudelli, A. C. Komor, H. A. Rees, M. S. Packer, A. H. Badran, D. I. Bryson and D. R. Liu, Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage, *Nature*, 2017, **551**(7681), 464–471.
- 49 Y. Xu and Z. Li, CRISPR-Cas Systems: Overview, Innovations and Applications in Human Disease Research and Gene Therapy, *Comput. Struct. Biotechnol. J.*, 2020, **18**, 2401–2415.
- 50 A. Rasheed, R. A. Gill, M. U. Hassan, A. Mahmood, S. Qari, Q. U. Zaman, M. Ilyas, M. Aamer, M. Batool, H. Li and Z. Wu, A Critical Review: Recent Advancements in the Use of Crispr/Cas9 Technology to Enhance Crops and Alleviate Global Food Crises, *Curr. Issues Mol. Biol.*, 2021, **43**(3), 1950–1976.
- 51 Y. Fu, J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon, J. K. Joung and J. D. Sander, High-Frequency off-Target Mutagenesis Induced by CRISPR-Cas Nucleases in Human Cells, *Nat. Biotechnol.*, 2013, **31**(9), 822–826.
- 52 Y. Mao, H. Zhang, N. Xu, B. Zhang, F. Gou and J. K. Zhu, Application of the CRISPR-Cas System for Efficient Genome Engineering in Plants, *Mol. Plant*, 2013, **6**(6), 2008–2011.
- 53 M. A. Nethery and R. Barrangou, Predicting and Visualizing Features of CRISPR-Cas Systems, *Methods Enzymol.*, 2019, **616**, 1–25.
- 54 M. A. Mengstie and B. Z. Wondimu, Mechanism and Applications of Crispr/Cas-9-Mediated Genome Editing, *Biol.: Targets Ther.*, 2021, **15**, 353–361.
- 55 M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity, *Science*, 2012, **337**(6096), 816–821.
- 56 G. Gasiunas, R. Barrangou, P. Horvath and V. Siksnys, Cas9-CrRNA Ribonucleoprotein Complex Mediates Specific DNA Cleavage for Adaptive Immunity in Bacteria, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**(39), E2579–E2586.
- 57 O. Rawashdeh, R. Y. Rawashdeh, T. Kebede, D. Kapp and A. Ralescu, Bio-Informatic Analysis of CRISPR Protospacer Adjacent Motifs (PAMs) in T4 Genome, *BMC Genomic Data*, 2022, **23**(1), 40.
- 58 J. A. Doudna and E. Charpentier, The New Frontier of Genome Engineering with CRISPR-Cas9, *Science*, 2014, **346**, 6213.
- 59 W. S. Ganpatrao and P. M. Baliram, Current and Future Prospects of Plant Breeding with CRISPR/Cas, *Curr. J. Appl. Sci. Technol.*, 2019, **1–17**, 2457–1024.
- 60 A. Macovei, N. R. Sevilla, C. Cantos, G. B. Jonson, I. Slamet-Loedin, T. Čermák, D. F. Voytas, I. R. Choi and P. Chadha-Mohanty, Novel Alleles of Rice EIF4G Generated by CRISPR/Cas9-Targeted Mutagenesis Confer Resistance to Rice Tungro Spherical Virus, *Plant Biotechnol. J.*, 2018, **16**(11), 1918–1927.
- 61 Y. Wang, X. Cheng, Q. Shan, Y. Zhang, J. Liu, C. Gao and J. L. Qiu, Simultaneous Editing of Three Homoeoalleles in Hexaploid Bread Wheat Confers Heritable Resistance to Powdery Mildew, *Nat. Biotechnol.*, 2014, **32**(9), 947–951.
- 62 M. Wang, Y. Lu, J. R. Botella, Y. Mao, K. Hua and J. K. Zhu, Gene Targeting by Homology-Directed Repair in Rice Using a Geminivirus-Based CRISPR/Cas9 System, *Mol. Plant*, 2017, **10**(7), 1007–1010.
- 63 A. C. Komor, Y. B. Kim, M. S. Packer, J. A. Zuris and D. R. Liu, Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage, *Nature*, 2016, **533**(7603), 420–424.
- 64 A. Kantor, M. E. McClements and R. E. MacLaren, CRISPR-Cas9 DNA base-editing and prime-editing, *Int. J. Mol. Sci.*, 2020, **21**(17), 6240.
- 65 A. C. Komor, A. H. Badran and D. R. Liu, CRISPR-based technologies for the manipulation of eukaryotic genomes, *Cell*, 2017, **168**(1), 20–36.
- 66 S. S. Bharat, S. Li, J. Li, L. Yan and L. Xia, Base editing in plants: current status and challenges, *Crop J.*, 2020, **8**(3), 384–395.
- 67 L. Alsøe, A. Sarno, S. Carracedo, D. Domanska, F. Dingler, L. Lirussi, T. SenGupta, B. N. Tekin, L. Jobert, B. L. Alexandrov, A. Galashevskaya, C. Rada, K. J. Sandve, T. Rongnes, E. H. Krokan and H. Nilsen, Uracil accumulation and mutagenesis dominated by cytosine deamination in CpG dinucleotides in mice lacking UNG and SMUG1, *Sci. Rep.*, 2017, **7**(1), 7199.
- 68 T. P. Huang, K. T. Zhao, S. M. Miller, N. M. Gaudelli, B. L. Oakes, C. Fellmann, F. D. Savage and D. R. Liu, Circularly permuted and PAM-modified Cas9 variants broaden the targeting scope of base editors, *Nat. Biotechnol.*, 2019, **37**(6), 626–631.
- 69 A. V. Anzalone, P. B. Randolph, J. R. Davis, A. A. Sousa, L. W. Koblan, J. M. Levy, J. P. Chen, C. Wilson, A. G. Newby, A. Raguram and D. R. Liu, Search-and-replace genome editing without double-strand breaks or donor DNA, *Nature*, 2019, **576**(7785), 149–157.
- 70 S. De Pater, J. E. Pinas, P. J. J. Hooykaas and B. J. van der Zaal, ZFN-Mediated Gene Targeting of the Arabidopsis Protoporphyrinogen Oxidase Gene through Agrobacterium-Mediated Floral Dip Transformation, *Plant Biotechnol. J.*, 2013, **11**(4), 510–515.
- 71 V. K. Shukla, Y. Doyon, J. C. Miller, R. C. Dekelver, E. A. Moehle, S. E. Worden, J. C. Mitchell, N. L. Arnold, S. Gopalan, X. Meng, V. M. Choi, J. M. Rock, Y. Y. Wu, G. E. Katibah, G. Zhifang, D. McCaskill, M. A. Simpson, B. Blakeslee, S. A. Greenwalt, H. J. Butler, S. J. Hinkley,



- L. Zhang, E. J. Rebar, P. D. Gregory and F. D. Urnov, Precise Genome Modification in the Crop Species *Zea Mays* Using Zinc-Finger Nucleases, *Nature*, 2009, **459**(7245), 437–441.
- 72 Y. Zhang, Q. Shan, Y. Wang, K. Chen, Z. Liang, J. Li, Y. Zhang, K. Zhang, J. Liu, D. F. Voytas, X. Zheng and C. Gao, Rapid and Efficient Gene Modification in Rice and *Brachypodium* Using TALENs, *Mol. Plant*, 2013, **6**(4), 1365–1368.
- 73 Y. Wang, X. Cheng, Q. Shan, Y. Zhang, J. Liu, C. Gao and J. L. Qiu, Simultaneous Editing of Three Homoeoalleles in Hexaploid Bread Wheat Confers Heritable Resistance to Powdery Mildew, *Nat. Biotechnol.*, 2014, **32**(9), 947–951.
- 74 W. Haun, A. Coffman, B. M. Clasen, Z. L. Demorest, A. Lowy, E. Ray, A. Retterath, T. Stoddard, A. Juillerat, F. Cedrone, L. Mathis, D. F. Voytas and F. Zhang, Improved Soybean Oil Quality by Targeted Mutagenesis of the Fatty Acid Desaturase 2 Gene Family, *Plant Biotechnol. J.*, 2014, **12**(7), 934–940.
- 75 T. Wendt, P. B. Holm, C. G. Starker, M. Christian, D. F. Voytas, H. Brinch-Pedersen and I. B. Holme, TAL Effector Nucleases Induce Mutations at a Pre-Selected Location in the Genome of Primary Barley Transformants, *Plant Mol. Biol.*, 2013, **83**(3), 279–285.
- 76 A. S. Fister, L. Landherr, S. N. Maximova and M. J. Guiltinan, Transient Expression of CRISPR/Cas9 Machinery Targeting TcNPR3 Enhances Defense Response in *Theobroma Cacao*, *Front. Plant Sci.*, 2018, **9**, 268.
- 77 V. Pompili, L. Dalla Costa, S. Piazza, M. Pindo and M. Malnoy, Reduced Fire Blight Susceptibility in Apple Cultivars Using a High-Efficiency CRISPR/Cas9-FLP/FRT-Based Gene Editing System, *Plant Biotechnol. J.*, 2020, **18**(3), 845–858.
- 78 J. Li, X. Meng, Y. Zong, K. Chen, H. Zhang, J. Liu, J. Li and C. Gao, Gene Replacements and Insertions in Rice by Intron Targeting Using CRISPR-Cas9, *Nat. Plants*, 2016, **2**(10), 16139.
- 79 A. Zhang, Y. Liu, F. Wang, T. Li, Z. Chen, D. Kong, J. Bi, F. Zhang, X. Luo, J. Wang, J. Tang, X. Yu, G. Liu and L. Luo, Enhanced Rice Salinity Tolerance via CRISPR/Cas9-Targeted Mutagenesis of the OsRR22 Gene, *Mol. Breed.*, 2019, **39**(3), 47.
- 80 X. Sun, X. Li, Y. Wang, J. Xu, S. Jiang and Y. Zhang, MdMCK9-Mediated the Regulation of Anthocyanin Synthesis in Red-Fleshed Apple in Response to Different Nitrogen Signals, *Int. J. Mol. Sci.*, 2022, **23**(14), 7755.
- 81 A. Endo, H. Saika, M. Takemura, N. Misawa and S. Toki, A Novel Approach to Carotenoid Accumulation in Rice Callus by Mimicking the Cauliflower Orange Mutation via Genome Editing, *Rice*, 2019, **12**(1), 81.
- 82 A. Souii, M. B. M'hadheb-Gharbi and J. Gharbi, Nucleic Acid-Based Biotechnologies for Food-Borne Pathogen Detection Using Routine Time-Intensive Culture-Based Methods and Fast Molecular Diagnostics, *Food Sci. Biotechnol.*, 2016, **25**, 11–20.
- 83 K. S. Gracias and J. L. McKillip, A Review of Conventional Detection and Enumeration Methods for Pathogenic Bacteria in Food, *Can. J. Microbiol.*, 2004, **50**, 883–890.
- 84 A. Pathologica, Rapid Detection, Characterization and Enumeration of Foodborne Pathogens, *J. Pathol. Microbiol. Immunol.*, 2011, **119**(s133), 1–24.
- 85 R. Patel, B. Mitra, M. Vinchurkar, A. Adami, R. Patkar, F. Giacomozzi, L. Lorenzelli and M. S. Baghini, A Review of Recent Advances in Plant-Pathogen Detection Systems, *Heliyon*, 2022, **8**(12).
- 86 B. Fraaije and D. Lovell, Septoria epidemics on wheat: combined use of visual assessment and PCR-based diagnostics to identify mechanisms of disease escape, *Plant Prot. Sci.*, 2017, **38**, 421–424.
- 87 A. C. Oluwaseun, P. Phazang and N. B. Sarin, Biosensors: A fast-growing technology for pathogen detection in agriculture and food sector, in *Biosensing Technologies for the Detection of Pathogens—A Prospective Way for Rapid Analysis*, 2018, pp. 37–52.
- 88 A. K. Gautam and S. Kumar, Techniques for the Detection, Identification, and Diagnosis of Agricultural Pathogens and Diseases, in *Natural Remedies for Pest, Disease and Weed Control*, Elsevier, 2020, pp. 135–142.
- 89 Y. Fang and R. P. Ramasamy, Current and Prospective Methods for Plant Disease Detection, *Biosensors*, 2015, **5**(3), 537–561.
- 90 G. Papadakis, N. Skandalis, A. Dimopoulou, P. Glynos and E. Gizeli, Bacteria Murmus; Application of an Acoustic Biosensor for Plant Pathogen Detection, *PLoS One*, 2015, **10**(7), e0132773.
- 91 N. P. Rijpens and L. M. F. Herman, Molecular Methods for Identification and Detection of Bacterial Food Pathogens, *J. AOAC Int.*, 2002, **85**(4), 984–995.
- 92 A. Souii, M. B. M'hadheb-Gharbi and J. Gharbi, Nucleic Acid-Based Biotechnologies for Food-Borne Pathogen Detection Using Routine Time-Intensive Culture-Based Methods and Fast Molecular Diagnostics, *Food Sci. Biotechnol.*, 2016, **25**, 11–20.
- 93 H. Y. Lau, H. Wu, E. J. H. Wee, M. Trau and J. R. Botella, Field Demonstration of Multiplexed Point-of-Care Diagnostic Platform for Plant Pathogens, *Anal. Chem.*, 2016, **88**(16), 8074–8081.
- 94 F. Zhan, T. Wang, L. Iradukunda and J. Zhan, A Gold Nanoparticle-Based Lateral Flow Biosensor for Sensitive Visual Detection of the Potato Late Blight Pathogen, *Phytophthora Infestans*, *Anal. Chim. Acta*, 2018, **1036**, 153–161.
- 95 Z. D. Chen, H. J. Kang, A. L. Chai, Y. X. Shi, X. W. Xie, L. Li and B. J. Li, Development of a Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of *Pseudomonas Syringae* Pv. Tomato in Planta, *Eur. J. Plant Pathol.*, 2020, **156**(3), 739–750.
- 96 X. Zong, W. Wang, H. Wei, J. Wang, X. Chen, L. Xu, D. Zhu, Y. Tan and Q. Liu, Rapid Detection of Prunus Necrotic Ringspot Virus Using Magnetic Nanoparticle-Assisted Reverse Transcription Loop-Mediated Isothermal Amplification, *J. Virol. Methods*, 2014, **208**, 85–89.
- 97 Y. Qiu, S. Zhang, A. Tan, *et al.*, Detection of TuMV by a toehold switch sensor coupled with NASBA



- amplification in *Pseudostellaria heterophylla*, *Plant Methods*, 2025, **21**, 81.
- 98 J. A. Ruemmele, W. P. Hall, L. K. Ruvuna and R. P. Van Duyn, A localized surface plasmon resonance imaging instrument for multiplexed biosensing, *Anal. Chem.*, 2013, **85**(9), 4560–4566.
- 99 A. Stals, L. Baert, E. Van Coillie and M. Uyttendaele, Extraction of Food-Borne Viruses from Food Samples: A Review, *Int. J. Food Microbiol.*, 2012, **153**, 1–9.
- 100 A. Stals, E. Van Coillie and M. Uyttendaele, Viral Genes Everywhere: Public Health Implications of PCR-Based Testing of Foods, *Curr. Opin. Virol.*, 2013, **3**(1), 69–73.
- 101 W. Sun, X. Huang and X. Wang, CRISPR-based molecular diagnostics: a review, *Shengwu Gongcheng Xuebao*, 2023, **39**(1), 60–73.
- 102 P. R. Shashank, B. M. Parker, S. R. Rananaware, D. Plotkin, C. Couch, L. G. Yang, L. T. Nguyen, N. R. Prasannakumar, W. E. Braswell, P. K. Jain and A. Y. Kawahara, CRISPR-Based Diagnostics Detects Invasive Insect Pests, *Mol. Ecol. Resour.*, 2023, **24**(1), e13881.
- 103 B. J. Hindson, K. D. Ness, D. A. Masquelier, P. Belgrader, N. J. Heredia, A. J. Makarewicz, I. J. Bright, M. Y. Lucero, A. L. Hiddessen, T. C. Legler, T. K. Kitano, M. R. Hodel, J. F. Petersen, P. W. Wyatt, E. R. Steenblock, P. H. Shah, L. J. Bousse, C. B. Troup, J. C. Mellen, D. K. Wittmann, N. G. Erndt, T. H. Cauley, R. T. Koehler, A. P. So, S. Dube, K. A. Rose, L. Montesclaros, S. Wang, D. P. Stumbo, S. P. Hodges, S. Romine, F. P. Milanovich, H. E. White, J. F. Regan, G. A. Karlin-Neumann, C. M. Hindson, S. Saxonov and B. W. Colston, High-throughput droplet digital PCR system for absolute quantitation of DNA copy number, *Anal. Chem.*, 2011, **83**(22), 8604–8610.
- 104 M. Z. Hossain and C. M. Maragos, Gold Nanoparticle-Enhanced Multiplexed Imaging Surface Plasmon Resonance (ISPR) Detection of Fusarium Mycotoxins in Wheat, *Biosens. Bioelectron.*, 2018, **101**, 245–252.
- 105 R. Takabatake, Y. Kagiya, S. Futo, Y. Minegishi, K. Soga, N. Shibata and K. Kondo, Rapid Screening Detection of Genetically Modified Papaya by Loop-Mediated Isothermal Amplification, *Biol. Pharm. Bull.*, 2023, **46**(5), 713–717.
- 106 Y. Zhou, H. Y. Zheng, D. M. Jiang, M. Liu, W. Zhang and J. Y. Yan, A Rapid Detection of Tomato Yellow Leaf Curl Virus Using Recombinase Polymerase Amplification-Lateral Flow Dipstick Assay, *Lett. Appl. Microbiol.*, 2022, **74**(5), 640–646.
- 107 M. K. Prasannakumar, P. B. Parivallal, D. Pramesh, H. B. Mahesh and E. Raj, LAMP-based foldable microdevice platform for the rapid detection of *Magnaporthe Oryzae* and *Sarocladium Oryzae* in rice seed, *Sci. Rep.*, 2021, **11**, 178.
- 108 S. B. Kokane, P. Misra, A. D. Kokane, *et al.*, Development of a real-time RT-PCR method for the detection of *Citrus tristeza virus* (CTV) and its implication in studying virus distribution in planta, *3 Biotech*, 2021, **11**, 431.
- 109 N. Datukishvili, T. Kutateladze, I. Gabriadze, K. Bitskinashvili and B. Vishnepolsky, New multiplex PCR methods for rapid screening of genetically modified organisms in foods, *Front. Microbiol.*, 2015, **6**, 757.
- 110 S. Wachiralurpan, I. Phung-On, N. Chanlek, S. Areekit, K. Chansiri and P. A. Lieberzeit, In-Situ Monitoring of Real-Time Loop-Mediated Isothermal Amplification with Qcm: Detecting *Listeria Monocytogenes*, *Biosensors*, 2021, **11**(9), 308.
- 111 M. Djisalov, L. Janjušević, V. Léguillier, L. Šašić Zorić, C. Farre, J. Anba-Mondoloni, J. Vidic and I. Gadjanski, Loop-Mediated Isothermal Amplification (LAMP) Assay Coupled with Gold Nanoparticles for Colorimetric Detection of *Trichoderma* Spp. in *Agaricus Bisporus* Cultivation Substrates, *Sci. Rep.*, 2024, **14**(1), 15539.
- 112 F. Jacquet, M. H. Jeuffroy, J. Jouan, E. Le Cadre, I. Litrico, T. Malausa, X. Reboud and C. Huyghe, Pesticide-Free Agriculture as a New Paradigm for Research, *Agron. Sustainable Dev.*, 2022, **42**, 8.
- 113 J. Zhang, H. Li, X. Zhong, J. Tian, A. Segers, L. Xia and F. Francis, RNA-Interference-Mediated Aphid Control in Crop Plants: A Review, *Agriculture*, 2022, **12**(12), 2108.
- 114 N. G. Bologna and O. Voinnet, The Diversity, Biogenesis, and Activities of Endogenous Silencing Small RNAs in Arabidopsis, *Annu. Rev. Plant Biol.*, 2014, **65**, 473–503.
- 115 M. W. Choudry, P. Nawaz, N. Jahan, R. Riaz, B. Ahmed, M. H. Raza, Z. Fayyaz, K. Malik and S. Afzal, RNA Based Gene Silencing Modalities to Control Insect and Fungal Plant Pests – Challenges and Future Prospects, *Physiol. Mol. Plant Pathol.*, 2024, **130**, 102241.
- 116 N. Agrawal, P. V. N. Dasaradhi, A. Mohammed, P. Malhotra, R. K. Bhatnagar and S. K. Mukherjee, RNA Interference: Biology, Mechanism, and Applications, *Microbiol. Mol. Biol. Rev.*, 2003, **67**(4), 657–685.
- 117 C. Cogoni, J. T. Irelan, M. Schumacher, T. J. Schmidhauser, E. U. Selker and G. Macino, Transgene Silencing of the Al-1 Gene in Vegetative Cells of *Neurospora* Is Mediated by a Cytoplasmic Effector and Does Not Depend on DNA-DNA Interactions or DNA Methylation, *EMBO J.*, 1996, **15**(12), 3153–3163.
- 118 F. L. Tan and J. Q. Yin, RNAi, a New Therapeutic Strategy against Viral Infection, *Cell Research*, 2004, **4**, 460–466.
- 119 A. Fire, *et al.*, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature*, 1998, **391**(6669), 806–811.
- 120 I. R. Henderson, X. Zhang, C. Lu, L. Johnson, B. C. Meyers, P. J. Green and S. E. Jacobsen, Dissecting Arabidopsis Thaliana DICER Function in Small RNA Processing, Gene Silencing and DNA Methylation Patterning, *Nat. Genet.*, 2006, **38**(6), 721–725.
- 121 E. J. Finnegan and M. A. Matzke, The Small RNA World, *J. Cell Sci.*, 2003, **116**(23), 4689–4693.
- 122 M. Ghildiyal and P. D. Zamore, Small Silencing RNAs: An Expanding Universe, *Nat. Rev. Genet.*, 2009, **10**, 94–108.
- 123 J. K. W. Lam, M. Y. T. Chow, Y. Zhang and S. W. S. Leung, siRNA versus miRNA as Therapeutics for Gene Silencing, *Mol. Ther.–Nucleic Acids*, 2015, **4**, e252.
- 124 F. Betti, M. J. Ladera-Carmona, D. A. Weits, G. Ferri, S. Iacopino, G. Novi, B. Svezia, A. B. Kunkowska,



- A. Santaniello, A. Piaggese, E. Loreti and P. Perata, Exogenous miRNAs Induce Post-Transcriptional Gene Silencing in Plants, *Nat. Plants*, 2021, 7(10), 1379–1388.
- 125 H. Sang and J. I. Kim, Advanced Strategies to Control Plant Pathogenic Fungi by Host-Induced Gene Silencing (HIGS) and Spray-Induced Gene Silencing (SIGS), *Agricultural Sciences*, 2020, 14, 1–8.
- 126 C. Rössner, D. Lotz and A. Becker, VIGS Goes Viral: How VIGS Transforms Our Understanding of Plant Science, *Annu. Rev. Plant Biol.*, 2025, 73, 703–728.
- 127 X. Chen, T. Shi, T. Tang, C. Chen, Y. Liang and S. Zuo, Nanosheet-Facilitated Spray Delivery of dsRNAs Represents a Potential Tool to Control *Rhizoctonia Solani* Infection, *Int. J. Mol. Sci.*, 2022, 23(21), 12922.
- 128 A. L. Jackson, J. Burchard, J. Schelter, B. N. Chau, M. Cleary, L. Lim and P. S. Linsley, Widespread siRNA “off-Target” Transcript Silencing Mediated by Seed Region Sequence Complementarity, *RNA*, 2006, 12(7), 1179–1187.
- 129 N. Mitter, E. A. Worrall, K. E. Robinson, P. Li, R. G. Jain, C. Taochy, S. J. Fletcher, B. J. Carroll, G. Q. Lu and Z. P. Xu, Clay Nanosheets for Topical Delivery of RNAi for Sustained Protection against Plant Viruses, *Nat. Plants*, 2017, 3(2), 1–10.
- 130 M. Knoblich, T. Gursinsky, S. Gago-Zachert, C. Weinholdt, J. Grau and S.-E. Behrens, A New Level of RNA-Based Plant Protection: DsRNAs Designed from Functionally Characterized siRNAs Highly Effective against *Cucumber Mosaic Virus*, *Nucleic Acids Res.*, 2025, 53(5), gkaf136.
- 131 K. A. Feldmann, Steroid regulation improves crop yield, *Nat. Biotechnol.*, 2006, 24(1), 46–47.
- 132 H. Hisano, R. Nandakumar and Z.-Y. Wang, Genetic Modification of Lignin Biosynthesis for Improved Biofuel Production, in *Biofuels: Global Impact on Renewable Energy, Production Agriculture, and Technological Advancements*, 2011, pp. 223–235.
- 133 F. Qiao, Q. Yang, C. L. Wang, Y. L. Fan, X. F. Wu and K. J. Zhao, Modification of Plant Height via RNAi Suppression of OsGA20ox2 Gene in Rice, *Euphytica*, 2007, 158(1–2), 35–45.
- 134 Y. Jiao, Y. Wang, D. Xue, J. Wang, M. Yan, G. Liu, G. Dong, D. Zeng, Z. Lu, X. Zhu, Q. Qian and J. Li, Regulation of OsSPL14 by OsmiR156 Defines Ideal Plant Architecture in Rice, *Nat. Genet.*, 2010, 42(6), 541–544.
- 135 K. Miura, M. Ikeda, A. Matsubara, X. J. Song, M. Ito, K. Asano, M. Matsuoka, H. Kitano and M. Ashikari, OsSPL14 Promotes Panicle Branching and Higher Grain Productivity in Rice, *Nat. Genet.*, 2010, 42(6), 545–549.
- 136 G. R. Davuluri, A. Van Tuinen, P. D. Fraser, A. Manfredonia, R. Newman, D. Burgess, D. A. Brummell, S. R. King, J. Palys, J. Uhlig, P. M. Bramley, H. M. J. Pennings and C. Bowler, Fruit-Specific RNAi-Mediated Suppression of DET1 Enhances Carotenoid and Flavonoid Content in Tomatoes, *Nat. Biotechnol.*, 2005, 23(7), 890–895.
- 137 B. Yu, D. J. Lydiate, L. W. Young, U. A. Schäfer and A. Hannoufa, Enhancing the Carotenoid Content of Brassica Napus Seeds by Downregulating Lycopene Epsilon Cyclase, *Transgenic Res.*, 2008, 17(4), 573–585.
- 138 A. M. Dandekar, G. Teo, B. G. Defilippi, S. L. Uratsu, A. J. Passey, A. A. Kader, J. R. Stow, R. J. Colgan and D. J. James, Effect of Down-Regulation of Ethylene Biosynthesis on Fruit Flavor Complex in Apple Fruit, *Transgenic Res.*, 2004, 13, 373–384.
- 139 A. Regina, A. Bird, D. Topping, S. Bowden, J. Freeman, T. Barsby, B. Kosar-Hashemi, Z. Li, S. Rahman and M. Morell, High-Amylose Wheat Generated by RNA Interference Improves Indices of Large-Bowel Health in Rats, *Agric. Sci.*, 2006, 103(10), 3546–3551.
- 140 J. Gil-Humanes, F. Pistón, C. M. Rosell and F. Barro, Significant Down-Regulation of γ -Gliadins Has Minor Effect on Gluten and Starch Properties of Bread Wheat, *J. Cereal Sci.*, 2012, 56(2), 161–170.
- 141 G. Chuai, H. Ma, J. Yan, M. Chen, N. Hong, D. Xue, C. Zhou, C. Zhu, K. Chen, B. Duan, F. Gu, S. Qu, D. Huang, J. Wei and Q. Liu, DeepCRISPR: Optimized CRISPR Guide RNA Design by Deep Learning, *Genome Biol.*, 2018, 19(1), 18.
- 142 T. J. Cradick, G. Ambrosini, C. Iseli, P. Bucher and A. P. McCaffrey, ZFN-Site Searches Genomes for Zinc Finger Nuclease Target Sites and off-Target Sites, *BMC Bioinf.*, 2011, 12, 152.
- 143 M. Stemmer, T. Thumberger, M. Del Sol Keyer, J. Wittbrodt and J. L. Mateo, CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool, *PLoS One*, 2015, 10(4), e0176619.
- 144 S. Lück, T. Kreszies, M. Strickert, P. Schweizer, M. Kuhlmann and D. Douchkov, siRNA-Finder (Si-Fi) Software for RNAi-Target Design and Off-Target Prediction, *Front. Plant Sci.*, 2019, 10, 1023.
- 145 G. Zhang, Z. Dai and X. Dai, C-RNNCrispr: Prediction of CRISPR/Cas9 SgRNA Activity Using Convolutional and Recurrent Neural Networks, *Comput. Struct. Biotechnol. J.*, 2020, 18, 344–354.
- 146 K. G. Ptitsyn, S. A. Khmeleva, L. K. Kurbatov, O. S. Timoshenko, E. V. Suprun, S. P. Radko and A. V. Lisitsa, LAMP Primer Designing Software: The Overview, *Biomed. Chem. Res. Methods*, 2024, 7(4), e00226.
- 147 R. Kalendar, A Guide to Using FASTPCR Software for PCR, In Silico PCR, and Oligonucleotide Analysis, in *PCR Primer Design*, ed. C. Basu, Springer US, New York, NY, 2021, pp. 223–243.
- 148 V. Periwal, A Comprehensive Overview of Computational Resources to Aid in Precision Genome Editing with Engineered Nucleases, *Briefings Bioinf.*, 2017, 18(4), 698–711.
- 149 A. Agmata, K. Labrador, J. D. Palermo and M. J. Pante, ThermoPlex: An Automated Design Tool for Target-Specific Multiplex PCR Primers Based on DNA Thermodynamics, *Biol. Methods Protoc.*, 2024, 10(1), bpaf074.
- 150 J. D. Limberis and J. Z. Metcalfe, PrimerJinn: A Tool for Rationally Designing Multiplex PCR Primer Sets for Amplicon Sequencing and Performing in Silico PCR, *BMC Bioinf.*, 2023, 24(1), 468.
- 151 L. Yang, F. Ding, Q. Lin, J. Xie, W. Fan, F. Dai, P. Cui and W. Liu, A Tool to Automatically Design Multiplex PCR



- Primer Pairs for Specific Targets Using Diverse Templates, *Sci. Rep.*, 2023, **13**(1), 16451.
- 152 H. Zeng, K. Chen, C. Ma, B. Zhu, J. Chuan, S. Zhang, L. Tang, T. Yang, Z. Sun, X. Yang and Y. Wang, High-Throughput Primer Design by Scoring in Piecewise Logistic Model for Multiple Polymerase Chain Reaction Variants, *Sci. Rep.*, 2022, **12**(1), 21136.
- 153 N. G. Xie, M. X. Wang, P. Song, S. Mao, Y. Wang, Y. Yang, J. Luo, S. Ren and D. Y. Zhang, Designing Highly Multiplex PCR Primer Sets with Simulated Annealing Design Using Dimer Likelihood Estimation (SADDLE), *Nat. Commun.*, 2022, **13**(1), 1881.
- 154 C. H. Yang, Y. H. Cheng, E. C. Yang, L. Y. Chuang and Y. Da. Lin, Multiobjective Optimization-Driven Primer Design Mechanism: Towards User-Specified Parameters of PCR Primer, *Briefings Bioinf.*, 2022, **23**(3), bbac121.
- 155 J. Yuan, J. Yi, M. Zhan, Q. Xie, T. T. Zhen, J. Zhou, Z. Li and Z. Li, The Web-Based Multiplex PCR Primer Design Software Ultiplex and the Associated Experimental Workflow: Up to 100- Plex Multiplicity, *BMC Genomics*, 2021, **22**(1), 835.
- 156 A. Kechin, V. Borobova, U. Boyarskikh, E. Khrapov, S. Subbotin and M. Filipenko, NGS-PrimerPlex: High-Throughput Primer Design for Multiplex Polymerase Chain Reactions, *PLoS Comput. Biol.*, 2020, **16**, e1008468.
- 157 H. Kim, N. N. Kang, K. H. An, J. H. Koo and M. S. Kim, MRPrimerW: A Tool for Rapid Design of Valid High-Quality Primers for Multiple Target QPCR Experiments, *Nucleic Acids Res.*, 2016, **44**(W1), W259–W266.
- 158 J. Bae, H. Jeon and M. S. Kim, GPrimer: A Fast GPU-Based Pipeline for Primer Design for QPCR Experiments, *BMC Bioinf.*, 2021, **22**(1), 220.
- 159 T. Zhu, C. Liang, Z. Meng, Y. Li, Y. Wu, S. Guo, R. Zhang, PrimerServer: A High-Throughput Primer Design and Specificity-Checking Platform, *bioRxiv*, 2017, preprint, DOI: [10.1101/181941](https://doi.org/10.1101/181941).
- 160 A. Xiao, Y. Wu, Z. Yang, Y. Hu, W. Wang, Y. Zhang, L. Kong, G. Gao, Z. Zhu, S. Lin and B. Zhang, EENdb: A Database and Knowledge Base of ZFNs and TALENs for Endonuclease Engineering, *Nucleic Acids Res.*, 2013, **41**(D1), D415–D422.
- 161 W. F. Lenoir, T. L. Lim and T. Hart, PICKLES: The Database of Pooled in-Vitro CRISPR Knockout Library Essentiality Screens, *Nucleic Acids Res.*, 2018, **46**(D1), D776–D780.
- 162 J. Park, J. S. Kim and S. Bae, Cas-Database: Web-Based Genome-Wide Guide RNA Library Design for Gene Knockout Screens Using CRISPR-Cas9, *Bioinformatics*, 2016, **32**(13), 2017–2023.
- 163 V. Jaskula-Ranga, D. J. Zack, GrID: A CRISPR-Cas9 Guide RNA Database and Resource for Genome-Editing, *bioRxiv*, 2016, preprint, DOI: [10.1101/097352](https://doi.org/10.1101/097352).
- 164 G. K. Varshney, S. Zhang, W. Pei, A. Adomako-Ankomah, J. Fohtung, K. Schaffer, B. Carrington, A. Maskeri, C. Slevin, T. Wolfsberg, J. Ledin, R. Sood and S. M. Burgess, CRISPRz: A Database of Zebrafish Validated SgRNAs, *Nucleic Acids Res.*, 2016, **44**(D1), D822–D826.
- 165 Y. Naito, K. Hino, H. Bono and K. Ui-Tei, CRISPRdirect: Software for Designing CRISPR/Cas Guide RNA with Reduced off-Target Sites, *Bioinformatics*, 2015, **31**(7), 1120–1123.
- 166 J. G. Mandell and C. F. Barbas, Zinc Finger Tools: Custom DNA-Binding Domains for Transcription Factors and Nucleases, *Nucleic Acids Res.*, 2006, **34**, W516–W523.
- 167 M. Jayakanthan, J. Muthukumar, S. Chandrasekar, K. Chawla, A. Punetha and D. Sundar, ZifBase: A Database of Zinc Finger Proteins and Associated Resources, *BMC Genomics*, 2009, **10**(1), 421.
- 168 J. D. Sander, M. L. Maeder, D. Reyon, D. F. Voytas, J. K. Joung and D. Dobbs, ZiFiT (Zinc Finger Targeter): An Updated Zinc Finger Engineering Tool, *Nucleic Acids Res.*, 2010, **38**, W462–W468.
- 169 E. L. Doyle, N. J. Booher, D. S. Standage, D. F. Voytas, V. P. Brendel, J. K. Vandyk and A. J. Bogdanove, TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: Tools for TAL Effector Design and Target Prediction, *Nucleic Acids Res.*, 2012, **40**, W117–W122.
- 170 J. Grau, J. Boch and S. Posch, TALENoffer: Genome-Wide TALEN off-Target Prediction, *Bioinformatics*, 2013, **29**(22), 2931–2932.
- 171 F. Heigwer, G. Kerr, N. Walther, K. Glaeser, O. Pelz, M. Breinig and M. Boutros, E-TALEN: A Web Tool to Design TALENs for Genome Engineering, *Nucleic Acids Res.*, 2013, **41**(20), e190.
- 172 Y. Lin, E. J. Fine, Z. Zheng, C. J. Antico, R. A. Voit, M. H. Porteus, T. J. Cradick and G. Bao, SAPTA: A New Design Tool for Improving TALE Nuclease Activity, *Nucleic Acids Res.*, 2014, **42**(6), e47.
- 173 M. Ma, A. Y. Ye, W. Zheng and L. Kong, A Guide RNA Sequence Design Platform for the CRISPR/Cas9 System for Model Organism Genomes, *BioMed Res. Int.*, 2013, **10**, 270805.
- 174 A. Xiao, Z. Cheng, L. Kong, Z. Zhu, S. Lin, G. Gao and B. Zhang, CasOT: A Genome-Wide Cas9/GRNA off-Target Searching Tool, *Bioinformatics*, 2014, **30**(8), 1180–1182.
- 175 S. Bae, J. Park and J. S. Kim, Cas-OFFinder: A Fast and Versatile Algorithm That Searches for Potential off-Target Sites of Cas9 RNA-Guided Endonucleases, *Bioinformatics*, 2014, **30**(10), 1473–1475.
- 176 S. J. Gratz, F. P. Ukken, C. D. Rubinstein, G. Thiede, L. K. Donohue, A. M. Cummings and K. M. Oconnor-Giles, Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in *Drosophila*, *Genetics*, 2014, **196**(4), 961–971.
- 177 F. Heigwer, G. Kerr and M. Boutros, E-CRISP: Fast CRISPR Target Site Identification, *Nat. Methods*, 2014, **11**, 122–123.
- 178 Y. Lei, L. Lu, H. Y. Liu, S. Li, F. Xing and L. L. Chen, CRISPR-P: A Web Tool for Synthetic Single-Guide RNA Design of CRISPR-System in Plants, *Mol. Plant*, 2014, **7**(9), 1494–1496.
- 179 A. O'Brien and T. L. Bailey, GT-Scan: Identifying Unique Genomic Targets, *Bioinformatics*, 2014, **30**(18), 2673–2675.
- 180 C. Ronda, L. E. Pedersen, H. G. Hansen, T. B. Kallehauge, M. J. Betenbaugh, A. T. Nielsen and H. F. Kildegaard, Accelerating Genome Editing in CHO Cells Using CRISPR



- Cas9 and CRISPy, a Web-Based Target Finding Tool, *Biotechnol. Bioeng.*, 2014, **111**, 1604–1616.
- 181 S. Xie, B. Shen, C. Zhang, X. Huang and Y. Zhang, SgRNAs9: A Software Package for Designing CRISPR SgRNA and Evaluating Potential off-Target Cleavage Sites, *PLoS One*, 2014, **9**(6), e100448.
- 182 L. J. Zhu, B. R. Holmes, N. Aronin and M. H. Brodsky, CRISPRseek: A Bioconductor Package to Identify Target-Specific Guide RNAs for CRISPR-Cas9 Genome-Editing Systems, *PLoS One*, 2014, **9**(9), e108424.
- 183 Y. Naito, K. Hino, H. Bono and K. Ui-Tei, CRISPRdirect: Software for Designing CRISPR/Cas Guide RNA with Reduced off-Target Sites, *Bioinformatics*, 2015, **31**(7), 1120–1123.
- 184 V. Pliatsika and I. Rigoutsos, “Off-Spotter”: Very Fast and Exhaustive Enumeration of Genomic Lookalikes for Designing CRISPR/Cas Guide RNAs, *Biol. Direct*, 2015, **10**(1), 4.
- 185 S. V. Prykhozhiy, V. Rajan, D. Gaston and J. N. Berman, CRISPR Multitargeter: A Web Tool to Find Common and Unique CRISPR Single Guide RNA Targets in a Set of Similar Sequences, *PLoS One*, 2015, **10**(3), e0138634.
- 186 K. Kaur, H. Tandon, A. K. Gupta and M. Kumar, CrisprGE: A Central Hub of CRISPR/Cas-Based Genome Editing, *Database*, 2015, **2015**, bav055.
- 187 A. Hodgkins, A. Farne, S. Perera, T. Grego, D. J. Parry-Smith, W. C. Skarnes and V. Iyer, WGE: A CRISPR Database for Genome Engineering, *Bioinformatics*, 2015, **31**(18), 3078–3080.
- 188 K. L. Neff, D. P. Argue, A. C. Ma, H. B. Lee, K. J. Clark and S. C. Ekker, Mojo Hand, a TALEN Design Tool for Genome Editing Applications, *BMC Bioinf.*, 2013, **14**(1), 1.
- 189 E. J. Fine, T. J. Cradick, C. L. Zhao, Y. Lin and G. Bao, An Online Bioinformatics Tool Predicts Zinc Finger and TALE Nuclease Off-Target Cleavage, *Nucleic Acids Res.*, 2014, **42**(6), e42.
- 190 F. Ahmed, M. Senthil-Kumar, X. Dai, V. S. Ramu, S. Lee, K. S. Mysore and P. X. Zhao, PssRNAit: A Web Server for Designing Effective and Specific Plant Sirnas with Genome-Wide off-Target Assessment1[OPEN], *Plant Physiol.*, 2020, **184**(1), 65–81.
- 191 A. Kakrana, R. Hammond, P. Patel, M. Nakano and B. C. Meyers, SPARTA: A Parallelized Pipeline for Integrated Analysis of Plant MiRNA and Cleaved MRNA Data Sets, Including New MiRNA Target-Identification Software, *Nucleic Acids Res.*, 2014, **42**(18), e139.
- 192 B. Liu, Y. Yuan, X. Pan, H.-B. Shen and C. Jin, AttSiOff: A Self-Attention-Based Approach on SiRNA Design with Inhibition and off-Target Effect Prediction, *Med-X*, 2024, **2**(1), 5.
- 193 R. Gumienny and M. Zavolan, Accurate Transcriptome-Wide Prediction of MicroRNA Targets and Small Interfering RNA off-Targets with MIRZA-G, *Nucleic Acids Res.*, 2015, **43**(3), 1380–1391.
- 194 R. Long, Z. Guo, D. Han, X. Yuan, G. Chen, P.-A. Heng, L. Zhang, SiRNA Design: A Graph Neural Network for SiRNA Efficacy Prediction via Deep RNA Sequence Analysis, *bioRxiv*, 2024, preprint, DOI: [10.1101/2024.04.28.591509](https://doi.org/10.1101/2024.04.28.591509).
- 195 Y. Chen, Y. Shi, Z. Wang, X. An, S. Wei, C. Andronis, J. Vontas, J. Wang and J. Niu, DsRNAEngineer: A Web-Based Tool of Comprehensive DsRNA Design for Pest Control, *Trends Biotechnol.*, 2024, **43**(4), 969–983.
- 196 W. Zhang, P. Zhang, W. Sun, J. Xu, L. Liao, Y. Cao and Y. Han, Improving Plant MiRNA-Target Prediction with Self-Supervised k-Mer Embedding and Spectral Graph Convolutional Neural Network, *PeerJ*, 2024, **12**(5), e17396.
- 197 J. Thody, V. Moulton, I. Mohorianu, PAREameters: Computational Inference of Plant MicroRNA-MRNA Targeting Rules Using RNA Sequencing Data, *bioRxiv*, 2019, preprint, DOI: [10.1101/710814](https://doi.org/10.1101/710814).
- 198 J. Thody, Computational Methods for Functional Analysis of Plant Small RNAs Using the RNA Degradome, *Doctoral thesis*, University of East Anglia, 2020, <https://ueaeprints.uea.ac.uk/id/eprint/79639>.
- 199 F. Hartung and J. Schiemann, Precise Plant Breeding Using New Genome Editing Techniques: Opportunities, Safety and Regulation in the EU, *Plant J.*, 2014, **78**(5), 742–752.
- 200 Scientific Opinion, Updated scientific opinion on plants developed through cisgenesis and intragenesis, *EFSA J.*, 2022, **20**(10), e07621.
- 201 E. Waltz, Tiptoeing around Transgenics. New techniques for manipulating plant genomes are yielding plants touted as non-transgenic. Will that relieve regulatory burden?, *Nat. Biotechnol.*, 2012, **30**(3), 215–217.
- 202 A. Kamthan, A. Chaudhuri, M. Kamthan and A. Datta, Small RNAs in Plants: Recent Development and Application for Crop Improvement, *Front. Plant Sci.*, 2015, **6**, 208.
- 203 P. Pant and F. Leese, Probing the Nucleic Acid Flexibility to Disarm the Viral Counter-Defense Machinery: Design and Characterization of Potent P19 Inhibitors, *J. Phys. Chem. B*, 2023, **127**(41), 8842–8851.

