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Gene cloning in vegetable improvement programme

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Abstract
The advent of genome-editing technology has enabled precise manipulation of DNA sequences within genomes, allowing for the targeted deletion or replacement of specific sequences in organisms, which results in intentional mutations. In plants, genome editing presents a compelling method for altering gene functions to develop enhanced crop varieties. This technology is considered user-friendly and poses a reduced risk of unintended off-target effects compared to traditional mutation breeding techniques. Moreover, genome-editing tools can be directly applied to crops with complex genomes or those that are challenging to breed through conventional methods. At present, highly adaptable genome-editing tools facilitate precise and predictable modifications at almost any locus in the plant genome, broadening their applications to include functional genomics research and molecular crop breeding. Vegetables, which are vital sources of nutrients for humans, supply essential vitamins, minerals, and fibre, thereby contributing significantly to human health. In this review, we provide a comprehensive overview of the evolution of genome-editing technologies, detailing the components of genome-editing toolkits and elucidating their fundamental mechanisms of action in representative systems. We discuss the current and potential applications of genome editing in developing more nutritious vegetables and present several case studies that demonstrate the technology's potential. Finally, we underscore future directions and challenges in utilizing genome-editing systems for vegetable crop research and product development.

Keywords: Gene cloning, genome-editing technology, precision breeding, transformation, vegetables

Introduction

Interest in the health benefits of consuming vegetables has surged due to their rich array of nutritional compounds, including vitamins, minerals, antioxidants, dietary fibre, and a diverse spectrum of phytochemicals (Septembre-Malaterre *et al.*, 2018) [70]. While vitamins and minerals are crucial for human health, antioxidant compounds found in fruits and vegetables are known to mitigate cellular oxidative stress and lower the risk of chronic diseases such as diabetes, cancer, and cardiovascular disorders (Aune *et al.*, 2017; Miller *et al.*, 2017) [8, 59]. Nevertheless, like all crops, vegetables are typically sensitive to biotic and abiotic stresses, making diseases, high temperatures, and limited water availability significant constraints on their productivity. These challenges are expected to intensify due to climate change (Bisbis *et al.*, 2018) [13]. Consequently, researchers are focused on enhancing vegetable varieties by improving yield and yield stability, nutritional quality, and tolerance to biotic and abiotic stresses through both traditional breeding methods and advanced plant molecular breeding technologies (Abdallah *et al.*, 2015) [1].

Plant breeding is a multifaceted process that involves developing new crop varieties with desirable traits and strategizing the combination of these traits to create superior varieties (Glenn *et al.*, 2017) [29]. The initial step in breeding involves leveraging genetic variation among individuals within a plant species. For many years, this has been accomplished by crossing parental lines or employing physical or chemical mutagenesis on crop plants (Holme *et al.*, 2019; Kleter *et al.*, 2019) [31, 42]. Since the early 20th century, when pioneering research discovered the ability of X-rays to alter plant phenotypes in crops such as barley and maize, a variety of innovative mutagenesis breeding tools have been developed (Muller, 1927; Stadler, 1928) [60, 76]. Subsequently, more advanced radiation techniques, including gamma radiation, UV light, and particle radiation, were introduced to generate novel agronomic traits and explore gene functions in crops (Shu *et al.*, 2012) [74].

Over time, chemical mutagens became preferred for their ease of use and higher mutation frequency compared to radiological methods. Today, ethyl methane sulfonate is the most widely used chemical mutagen, with other agents like sodium azide and methyl nitrosourea (Az-MNU) also commonly employed for mutagenesis in plants.

Physical and chemical mutagenesis has been effectively utilized to induce point mutations and deletions within plant genomes, leading to the release of thousands of varieties developed through mutation breeding (Ahloowalia *et al.*, 2004) [2]. While the potential of mutagenesis in vegetable breeding has not been fully realized, it has found extensive application in generating variation in ornamental plants (Bernardo, 2016) [10]. With the advancement of molecular technologies and DNA sequencing, researchers began investigating the functional properties of genes using T-DNA-tagged mutant pools created through random T-DNA insertional mutagenesis. This approach allows for the identification of DNA sequences flanking T-DNA, facilitating the exploration of genetic elements potentially responsible for phenotypic changes in mutants. The data gathered from analyzing T-DNA flanking sequences has been pivotal, providing new avenues for developing improved crop varieties with desirable traits using molecular breeding tools (Chaudhary *et al.*, 2019; Kleter *et al.*, 2019) [20, 42]. In recent decades, significant advancements have been made in molecular biology techniques. The discovery of sequence-specific nucleases (SSNs) and the development of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system have enabled precise, programmable gene editing at the DNA level, allowing for the creation of vegetable crops with modified functions and desired traits (Abdallah *et al.*, 2015; Nunez de Caceres Gonzalez and De la Mora Franco, 2020) [15, 61].

History of gene cloning/gene-editing technology

In recent years, the advancement of gene and genome-editing technologies has significantly enhanced the precise and efficient modification of genomes across various organisms, including vegetable crops, to improve yield and quality (Chen and Gao, 2014) [21]. Sophisticated molecular biology techniques utilizing sequence-specific nucleases (SSNs), such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR-Cas system (Chen and Gao, 2014) [21], have empowered plant researchers to perform targeted gene and genome engineering with remarkable precision and efficiency. These methods induce double-strand breaks (DSBs) at specific DNA loci, which are subsequently repaired by the cell's endogenous DNA repair mechanisms. This repair can either involve the insertion or deletion of nucleotides through non-homologous end joining (NHEJ) or gene replacement via homologous recombination (HR), thereby causing loss-of-function or gain-of-function in target genes (Symington and Gautier, 2011; Figure 1) [79]. The various CRISPR-Cas systems, in particular, have emerged as powerful tools for gene and genome editing, leading to the creation of numerous genome-edited plants using these technologies (Gao, 2014) [27].

Zinc-Finger Nucleases

Zinc-finger nucleases (ZFNs), the first generation of site-specific nucleases, enabled rapid and targeted modifications of the genome. ZFNs are typically constructed by fusing zinc-finger protein (ZFP) domains, which can bind to DNA sequences in a sequence-specific manner, with a nonspecific DNA cleavage domain derived from the bacterial FokI endonuclease (Figure 1; Petolino, 2015) [63]. Each ZFP, consisting of a tandem array of cysteine2 and histidine2 (Cys2-His2) domains (Miller *et al.*, 1985) [58], recognizes approximately three base pairs (bp) of DNA. Generally, four to six ZFPs are linked together to recognize a specific DNA sequence of 12 to 18 bp (Urnov *et al.*, 2010) [83]. For DNA cleavage to occur, the FokI catalytic domain must dimerize, meaning ZFNs function as heterodimers to target and cut DNA. Once the zinc-finger domain identifies and binds to the target DNA, the attached FokI nuclease domain cleaves the DNA, creating double-strand breaks (DSBs) at the targeted locus (Lee *et al.*, 2016) [43]. ZFNs have been utilized to modify specific gene sequences in various crops, including maize (Shukla *et al.*, 2009; Ainley *et al.*, 2013) [75, 3], soybean (Curtin *et al.*, 2011) [24], rice (Cantos *et al.*, 2014; Jung *et al.*, 2018) [18, 39], and apple (Peer *et al.*, 2015) [62].

Transcription Activator-Like Effector Nucleases

Transcription activator-like effector nucleases (TALENs) have emerged as an alternative to zinc-finger nucleases (ZFNs) for genome editing (Sakuma and Yamamoto, 2017) [67]. Like ZFNs, TALENs are composed of DNA-binding and nuclease domains derived from the FokI enzyme. The DNA-binding domain in TALENs, known as transcription activator-like effector (TALE) repeats, is typically made up of repetitive sequences derived from TALEs secreted by Xanthomonas bacteria, which facilitate the infection of plant species (Figure 1). Each TALE repeat is designed to recognize a single nucleotide within a genomic sequence (Joung and Sander, 2013) [38]. A single DNA-binding repeat consists of a conserved 34-amino acid sequence with a highly variable region at positions 12 and 13, referred to as the repeat variable di-residue (RVD), which determines the nucleotide-binding specificity (Richter *et al.*, 2020) [65]. By combining specific RVDs, a TALE DNA-binding domain, linked to the FokI nuclease domain, can generate double-strand breaks (DSBs) at a desired genomic target site. Similar to ZFNs, TALEN-mediated genome editing has been successfully employed for targeted mutagenesis in various crop species, including rice (Shan *et al.*, 2013) [71], soybean (Haun *et al.*, 2014) [30] and potato (Sawai *et al.*, 2014) [68].

Precise genome editing in plants

Targeted mutagenesis in plants primarily relies on non-homologous end joining (NHEJ) to repair double-strand breaks (DSBs). This repair mechanism is inherently error-prone, often resulting in small deletions, insertions, or nucleotide substitutions (Atkins and Voytas, 2020) [7]. In contrast, homology-directed repair (HDR) is a less frequent pathway due to its lower editing efficiency. However, HDR allows for precise gene insertions or replacements when a suitable repair template is provided.

The advent of modern mutagenesis tools, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR-Cas systems, has significantly transformed the landscape of plant molecular biology and functional genomics. These tools hold considerable promise for advancing crop development through the targeted modification of agronomic traits (Chen *et al.*, 2019) [22]. Although ZFNs and TALENs have been instrumental in targeted mutagenesis across various plant species such as *Arabidopsis*, maize, Brassica, rice, barley, soybean, tobacco, tomato, wheat, potato, and sugarcane (Shelake *et al.*, 2019) [72], the CRISPR-Cas system has emerged as the most widely adopted genome editing tool. Its popularity is attributed to its straightforward engineering process, versatility,

affordability, high efficiency, and remarkable specificity (Brandt and Barrangou, 2019) [15]. This system shows immense potential for genome editing aimed at developing improved cultivars with added value (Zhang *et al.*, 2020c) [88].

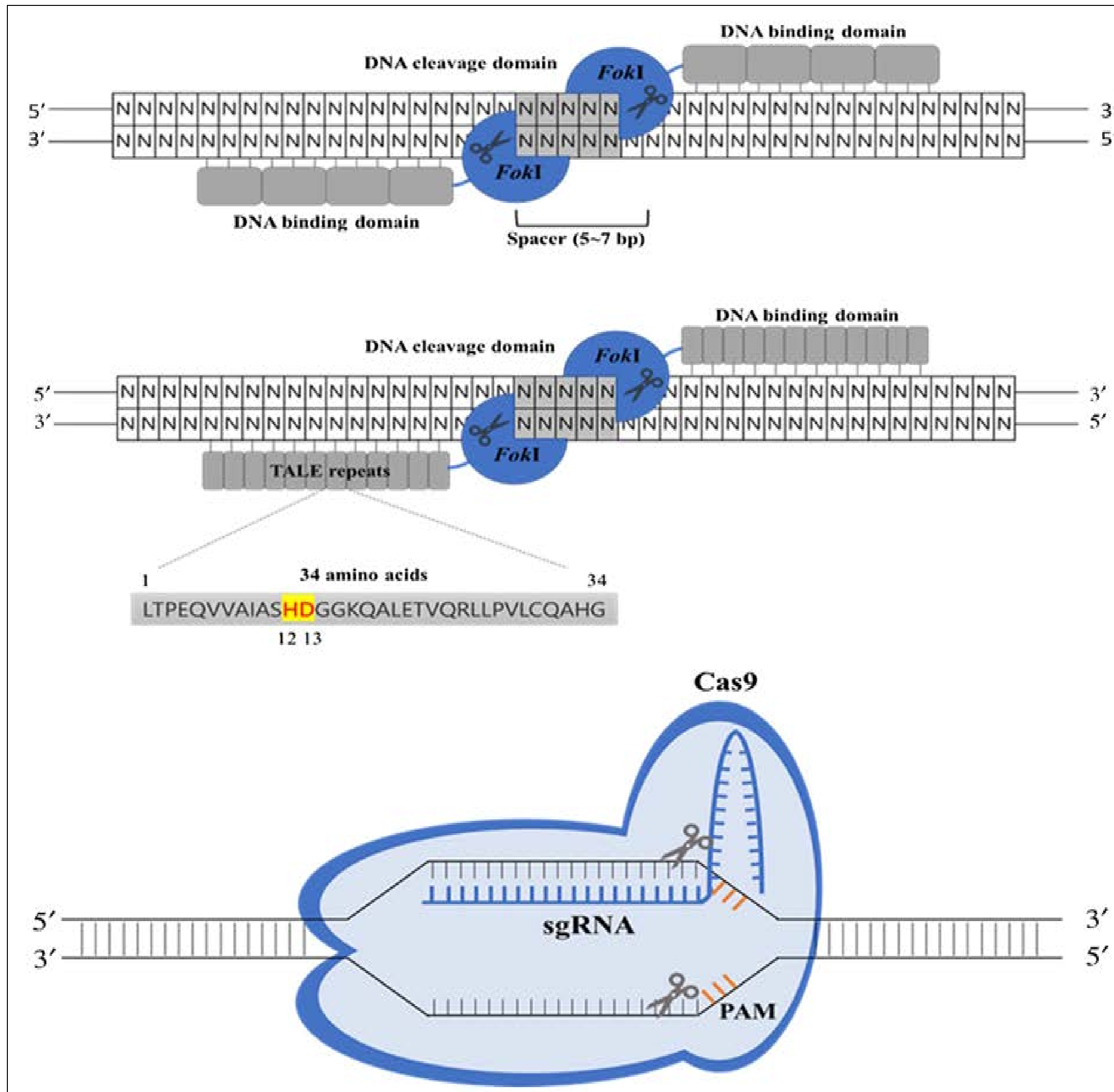

Fig 1: Some of the major genome-editing technologies using site-specific nucleases include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR-Cas9) systems.

Basic CRISPR-Cas System

The CRISPR-Cas9 system has become a widely used platform for genome engineering, primarily due to its simplicity, versatility, and precision (Figure 2). The system's central component, Cas9 (SpCas9), originates from the type II CRISPR-Cas Class 2 system of Streptococcus pyogenes. It was the first nuclease adapted for genome editing and remains the most frequently utilized enzyme in this domain (Mali *et al.*, 2013) [56]. The CRISPR-Cas9 system functions with the help of a single-guide RNA (sgRNA), designed to operate in the crRNA-tracrRNA complex. The sgRNA guides the Cas9 protein to specific genomic loci through complementary base pairing between the sgRNA and the target DNA sequence, located next to a protospacer adjacent motif (PAM). The Cas9 protein then cleaves both DNA strands, typically generating blunt-end cuts three base pairs upstream of the PAM site (5'-NGG; Makarova *et al.*, 2015) [55].

To overcome the limited availability of target sites recognized by SpCas9, various Cas9 variants have been developed to recognize different PAM sequences. These include xCas9, which recognizes 5'-NG, 5'-GAA, and 5'-GAT (Hu *et al.*, 2018) [34]; *Staphylococcus aureus* Cas9 (SaCas9), which recognizes 5'-NNGRRT; *Streptococcus thermophilus* Cas9 (StCas9), which recognizes 5'-NNAGAAW (Cong *et al.*, 2013) [23]; *Neisseria meningitidis* Cas9 (NmCas9), which recognizes 5'-NNNNGATT (Esvelt *et al.*, 2013) [25]; *Campylobacter jejuni* Cas9 (CjCas9), which recognizes 5'-NNNVRYM (Yamada *et al.*, 2017) [85]; and CasX, also known as Cas12e, which recognizes 5'-TTCN (Burstein *et al.*, 2017) [17]. Additionally, Cas13 variants, which target RNA instead of DNA, have been employed to study virus biology in plants, showcasing high interference activities against RNA viruses (Mahas *et al.*, 2019) [54]. These innovations have significantly expanded the flexibility and applicability of CRISPR-Cas systems, enabling targeted genetic modifications across a broad spectrum of organisms, including plants, for enhanced trait development and disease resistance.

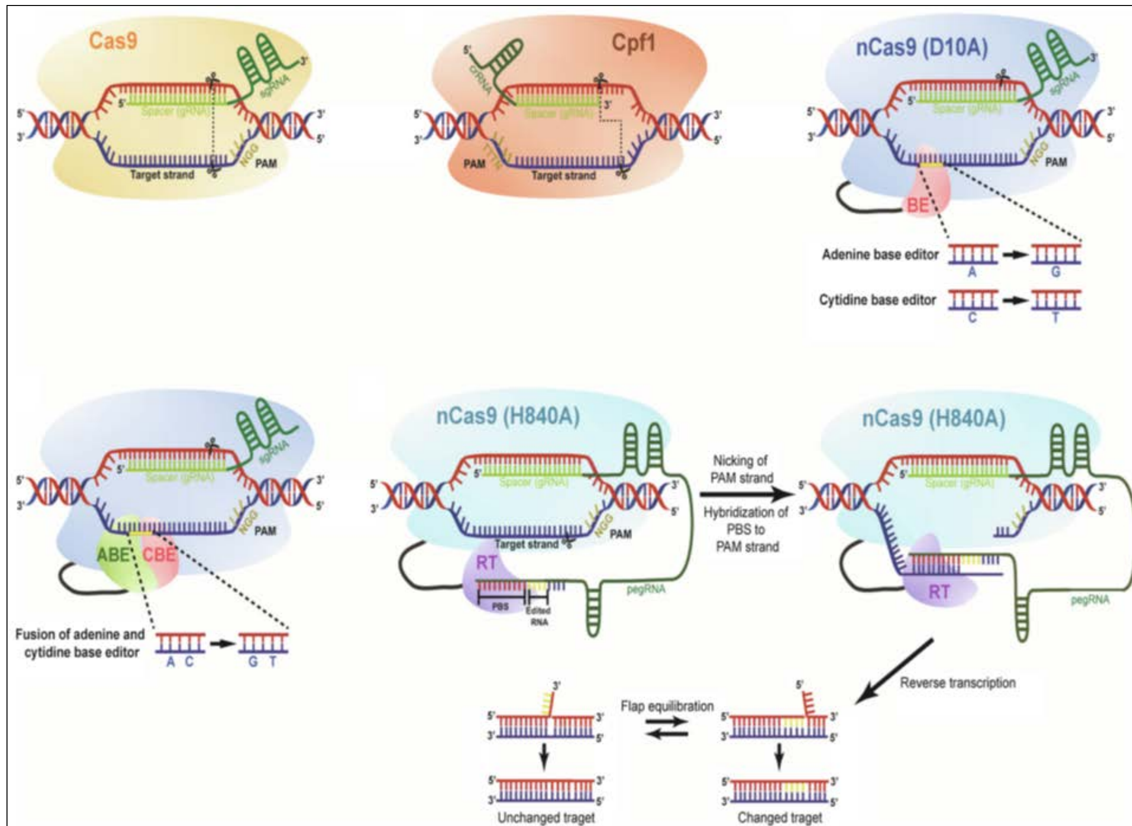

Fig 2: Diagram of the CRISPR-Cas9

Base Editing

Deaminase-mediated base editing technology is an advanced genome-editing tool that enables precise point mutations in target genomic regions without inducing double-strand breaks (DSBs). This technology involves a base editor fused to a Cas9 nickase, which is directed to a specific DNA sequence by a guide RNA (gRNA). Two primary base editing tools, cytosine base editors (CBEs) and adenine base editors (ABEs), have been developed for this purpose (Shimatani *et al.*, 2017;) [73]. Furthermore, dual base editor-mediated precise genome-editing technology has also been applied to plants.

Cytosine Base Editors (CBEs) consist of a Cas9 nickase (nCas9) with a D10A mutation that inactivates one of the nucleolytic domains, RuvC, of Cas9. This nCas9 is fused with a cytidine deaminase and a uracil glycosylase inhibitor (UGI; Figure 2C). The cytidine deaminase catalyzes the deamination of cytidine in the DNA strand, converting it into uridine. When the nCas9 (D10A) protein nicks the non-target DNA strand, the U-G mismatch induces a T-A substitution in the resulting DNA strands through DNA repair and replication processes (C-to-T conversion). CBE-mediated base editing has been effectively employed in various crops such as rice, maize, wheat, and potato, showing relatively high efficiency (Shimatani *et al.*, 2017; Zong *et al.*, 2017) [73, 92].

Adenine Base Editors (ABEs), another class of base editing technology, facilitate the conversion of A-T base pairs to G-C. Adenine base editors utilize an adenosine deaminase fused with nCas9 (D10A), which deaminates adenosine to inosine. Inosine pairs with cytidine and is interpreted as guanine by DNA polymerase during the DNA repair and replication process, leading to an A-T to G-C conversion (Gaudelli *et al.*, 2017) [28]. ABEs have been applied in crops like Arabidopsis, wheat, rice, and rapeseed, although the efficiency is generally lower compared to the original SpCas9 or SaCas9 systems (Kang *et al.*, 2018) [40]. However, recent development of ABE variants in mammalian cells could potentially enhance editing efficiency in plants (Richter *et al.*, 2020) [65].

Allows simultaneous conversion of C-G to T-A and A-T to G-C. This method utilizes the fusion of nCas9 with both a CBE and an ABE, a system known as the Saturated Targeted Endogenous Mutagenesis Editor (STEME). This tool enables the generation of dual base substitutions (C-G to T-A and A-T to G-C) using a single guide RNA (sgRNA). The STEME system, which comprises an ABE, a CBE, nCas9 (D10A), sgRNA, and UGI, was recently used for genome editing in rice (Richter *et al.*, 2020) [65]. STEMEs can produce a diverse array of mutations, including base substitutions and in-frame insertions and deletions (indels) with high efficiency, making dual base editing particularly useful for studying cis-elements in noncoding regions and for genome-wide screening of cis-regulatory regions.

Prime Editing

One of the primary challenges with current genome-editing tools is their technical complexity and extremely low efficiency in introducing customized sequences at specific target sites. To address this limitation, Anzalone *et al.* (2019) [5] developed a ground-breaking genome-editing technology called prime editing. This innovative approach enables 12 types of base conversions in target genes at locations ranging from 3 base pairs (bp) upstream to 29 bp downstream of the protospacer adjacent motif (PAM). Prime editing allows for precise insertions of up to 44 bp and deletions of up to 80 bp, all without causing double-strand breaks (DSBs) (Anzalone *et al.*, 2019) [5].

The prime editing system is composed of a Cas9 nickase (nCas9) with an H840A mutation fused to a reverse transcriptase (RTase) and a prime editing guide RNA (pegRNA). The pegRNA directs the nCas9 (H840A) to bind to the target DNA sequence and consists of two key regions: a spacer that is complementary to the non-edited DNA strand at the 5' end of the

pegRNA, and a prime binding site (PBS) located at the 3' end of the pegRNA. The PBS is crucial for recognizing the sequence of the DNA strand to be edited and includes the desired sequences to be introduced into the target site (Figure 2E). In this system, the PBS functions as a primer for the RTase linked to nCas9 (H840A). The RTase uses the pegRNA as a template, which pairs with the nCas9 (H840A)-nicked single-stranded DNA (ssDNA) strand, effectively copying the genetic information from the pegRNA into the target genome site (Anzalone *et al.*, 2019) [5]. After reverse transcription, the system undergoes equilibration between the 3' edited DNA flap and the 5' unedited DNA flap, followed by the integration of the edited DNA into the target site via ligation and the DNA repair system (Anzalone *et al.*, 2019) [5].

Despite its potential, the prime editing system's efficiency in plants has been limited, and its ability to achieve precise gene editing has only been verified in two food crops: rice and wheat (Lin *et al.*, 2020) [50]. Therefore, additional studies are required to demonstrate the applicability of prime editing technology in a wider range of organisms. These studies will be crucial for advancing the use of prime editing tools and making continuous improvements to the technology, thereby expanding its utility in various fields of genetic research and crop development.

Chromosome Engineering

In plant breeding, the goal is to combine numerous favourable traits into a single breeding line. Since traits are determined by genes located on chromosomes, genes that are located close to each other on the same chromosome are often inherited together, a phenomenon known as complete linkage. However, this linkage can sometimes include unfavourable traits, making it challenging to isolate and maintain beneficial traits through traditional breeding methods. To address this issue, CRISPR-Cas9 technology offers a promising solution by enabling precise genome editing. The technology can induce double-strand breaks (DSBs) in DNA, which, when followed by homologous recombination (HR), can potentially disrupt unwanted linkages and facilitate the separation of target genes from linked unfavourable traits (Filler Hayut *et al.*, 2017) [26]. Despite this potential, the frequency of crossover (CO) events that lead to the separation of linked genes remains relatively low, and efficient detection systems are required to identify successful HR events. Early studies demonstrated that CRISPR-Cas9-mediated DSB induction could lead to DNA fragment inversions in plants, a technique that has been used to revert large inversions and thus make the region accessible to meiotic crossovers (Zhang *et al.*, 2017) [86]. This approach has allowed for the elimination of unwanted inversions and has facilitated the recombination of desirable traits. For instance, research has shown that the introduction of multiple DSBs, although with low efficiency, can cause inversions, and the reversal of a 1.1 Mb inversion on Arabidopsis chromosome 4 using SaCas9 has been successfully achieved (Schmidt *et al.*, 2020) [69]. Moreover, CRISPR-Cas9 technology has enabled the creation of heritable reciprocal translocations in the mega base pair (Mbp) range between nonallelic chromosomes, a process that has significant implications for plant breeding (Beying *et al.*, 2020) [12]. This approach could substantially enhance the introgression of genes from wild relatives into cultivated varieties by reducing linkage drag, thereby making extensive backcrossing less necessary.

Gene cloning strategies for vegetables with improved traits

Vegetables play a crucial role in human nutrition due to their rich content of essential nutrients and phytochemicals, which are vital for disease prevention and overall health maintenance. To reduce the risk of cardiovascular diseases and cancer, recent guidelines recommend consuming over 400 grams of vegetables and fruits daily (Aune *et al.*, 2017) [8]. However, vegetable crops face numerous challenges, including susceptibility to pests, diseases caused by viruses, bacteria, and fungi, and abiotic stresses such as drought, salinity, flooding, and nutrient deficiencies (Boscaiu and Fita, 2020) [14]. To address these challenges, breeding programs aim to develop vegetable varieties that are resistant to biotic stresses, tolerant to abiotic conditions, and high yielding with enhanced nutritional content. In this context, CRISPR-Cas9 technology has emerged as a promising tool to achieve these goals (Khatodia *et al.*, 2017) [41]. CRISPR-Cas9 allows for precise genome editing, which can be utilized to enhance resistance to pests and diseases, improve stress tolerance, and boost nutritional qualities in vegetable crops. By enabling targeted modifications in the plant genome, CRISPR-Cas9 can help create varieties that better withstand environmental challenges and provide greater health benefits to consumers (Table 1).

Tomato

Tomato (*Solanum lycopersicum* L.) serves as a prominent model for testing CRISPR-Cas9 approaches due to its well-characterized genome and significant economic value. Since the advent of CRISPR-Cas9 in tomato research, several notable advancements have been made:

1. **Initial Applications:** The first successful CRISPR-Cas9-mediated genome editing in tomato was reported in 2014. Researchers targeted the SIAGAMOUS-LIKE 6 (SIAGL6) gene, which is involved in leaf development, demonstrating the tool's potential in manipulating developmental pathways (Brooks *et al.*, 2014) [16].
2. **Fruit Ripening:** The CRISPR-Cas9 system was further applied to the RIPENING INHIBITOR (RIN) gene, which encodes a MADS-domain transcription factor crucial for fruit ripening. Mutations in RIN resulted in incomplete fruit ripening, underscoring the gene's critical role and the potential for CRISPR-Cas9 to influence fruit quality (Ito *et al.*, 2015) [37].
3. **Targeted Mutagenesis and Marker-Free Editing:** Shimatani *et al.* (2017) [73] employed a base-editing approach known as Target-AID (CRISPR-AID), which combines a Cas9 nickase (nCas9, D10A) with a cytidine deaminase. This method was used to create marker-free, homozygous DNA substitutions in endogenous tomato genes, DELLA (Solyc11g011260) and Ethylene Resistance 1 (ETR1; Solyc12g011330), which are involved in plant hormone signalling (Shimatani *et al.*, 2017) [73].
4. **Stress Tolerance:** Tomato plants with CRISPR-Cas9-induced mutations in the Solanum lycopersicum mitogen-activated protein kinase 3 (slmapk3) gene exhibited enhanced tolerance to heat stress. These plants showed reduced wilting, less membrane damage, lower reactive oxygen species (ROS) levels, and increased antioxidant enzyme activity, highlighting the utility of CRISPR-Cas9 for improving stress resilience.
5. **Dwarfism and Gibberellin Regulation:** In 2019, Tomlinson *et al.* reported the use of CRISPR technology to create a dominant dwarf mutation by modifying the PROCERA gene, which encodes a DELLA protein. DELLA proteins are negative regulators of gibberellin signalling and altering this gene can lead to desirable dwarfing traits in tomato plants (Tomlinson *et al.*, 2019) [83].

Cucumber

Cucumber (*Cucumis sativus* L.), a member of the Cucurbitaceae family, is a widely cultivated vegetable with significant economic value across temperate and tropical regions. The CRISPR-Cas9 system has been utilized to advance cucumber breeding and improve traits such as disease resistance and flowering characteristics:

- **Viral Resistance:** The initial application of CRISPR-Cas9 in cucumber focused on enhancing viral resistance. By targeting the eukaryotic translation initiation factor 4E (eIF4E) gene, researchers successfully conferred broad-spectrum resistance to various viral infections. This approach highlights CRISPR-Cas9's potential for developing crops with improved disease resistance (Chandrasekaran *et al.*, 2016) [19].
- **Gynoecious Phenotype:** In cucumber breeding, gynoecious lines are valuable due to their higher fruit production and reduced labour costs for crossing. Hu *et al.* (2017) [32] employed CRISPR-Cas9 to generate mutants of the WPP trp/pro/pro domain Interacting Protein1 (CsWIP1) gene, which encodes a zinc-finger transcription factor involved in flower development. The resulting Cswip1 T0 mutants displayed a gynoecious phenotype, bearing only female flowers. This result indicates that CsWIP1 plays a role in suppressing cucumber carpel development, making it a key target for manipulating flowering traits (Hu *et al.*, 2017) [32].

Watermelon

Watermelon (*Citrullus lanatus*), a member of the Cucurbitaceae family, is valued for its nutritional content, including citrulline, vitamins, and lycopene (Maoto *et al.*, 2019) [57]. The CRISPR-Cas9 system has been instrumental in watermelon genetic modification, with several applications aimed at improving fruit characteristics and resistance to pests and diseases:

1. **Albino Phenotype:** The CRISPR-Cas9 system was used to target the phytoene desaturase (ClPDS) gene, which encodes an essential enzyme in carotenoid biosynthesis. Mutations in this gene resulted in an albino phenotype, demonstrating the system's efficacy in altering pigment production (Tian *et al.*, 2017) [81].
2. **Herbicide Resistance:** Base editing technology was applied to the acetolactate synthase (ClALS) gene, achieving single-nucleotide conversions that conferred resistance to sulfonylurea herbicides. Watermelon plants with C to T mutations at Pro 190 in the ClALS gene were resistant to these herbicides while maintaining fruit and seed size and yield (Tian *et al.*, 2018) [80].
3. **Disease Resistance:** The CRISPR-Cas9 system was employed to knockout the phytosulfokine1 (ClPSK1) gene, which is involved in susceptibility to *Fusarium oxysporum* f. sp. *niveum* (FON). The loss-of-function mutations in ClPSK1 resulted in increased resistance to FON, enhancing plant resilience to this pathogen (Zhang *et al.*, 2020b) [88].
4. **Gynoecious Lines:** The ClWIP1 gene, a homologue of CsWIP1 in cucumber and CmWIP1 in melon, was targeted to create gynoecious watermelon lines. ClWIP1 is involved in carpel development and its mutation led to the production of lines with a gynoecious phenotype, showing its role in floral development (Zhang *et al.*, 2020a) [88].

Eggplant

Eggplant (*Solanum melongena* L.), the fifth most produced vegetable globally with 52.3 million tons in 2017 (Alam and Salimullah, 2021) [4], faces challenges such as enzymatic browning, which affects the quality and marketability of its fruits. Polyphenol oxidase (PPO) enzymes are primarily responsible for this browning process. In eggplant, three PPO genes-SmelPPO4, SmelPPO5, and SmelPPO6-have been identified as having high transcript levels in fruit after cutting, indicating their significant role in enzymatic browning. To address this issue, researchers have utilized CRISPR-Cas9-based mutagenesis to simultaneously knockout these three PPO genes. This approach aims to reduce or eliminate the browning of eggplant fruit flesh, thereby improving its quality and shelf life. The application of CRISPR-Cas9 technology in this context demonstrates its potential to enhance the post-harvest characteristics of eggplants by targeting specific genes involved in undesirable traits, such as enzymatic browning.

Leafy vegetable

Leafy vegetables have benefited from CRISPR-Cas applications for trait improvement, including the development of methods for precise genome editing without the integration of foreign DNA. In lettuce and cabbage, for example, CRISPR-Cas9 and Cpf1 (Now known as Cas12a) RNPs (Ribonucleoprotein complexes) have been directly delivered into protoplasts-a method known as a DNA-free genome-editing approach. Woo *et al.* (2015) [84] demonstrated successful delivery of CRISPR-Cas9 RNPs into lettuce protoplasts using PEG-mediated transfection. This technique led to the regeneration of plants with the intended mutations. More recent studies suggest that electroporation may offer improved efficiency over PEG-mediated methods for delivering RNPs into cabbage protoplasts. Electroporation is believed to have lower chemical toxicity, which may enhance its effectiveness for genome editing in leafy vegetables (Lee *et al.*, 2020) [44]. Additionally, genome editing has been applied to modulate the translation of mRNA in leafy vegetables. Editing the upstream open reading frame (uORF) of genes involved in vitamin C biosynthesis, such as LsGGP1 and LsGGP2, has shown promising results. By disrupting the uORF, researchers increased mRNA translation efficiency, leading to elevated ascorbate content and improved tolerance to oxidative stress. This demonstrates the potential of CRISPR-Cas9 technology to enhance nutritional quality and stress resilience in leafy vegetables.

Perspectives

The debate over the regulation of new gene-editing techniques is indeed critical, especially given the potential benefits these technologies offer in crop improvement. While strict regulatory policies, like those in the EU, might limit the application of these innovations, recent reports from the European Commission suggest a cautious optimism about their potential. The advancement of more efficient and precise gene-editing systems, alongside the integration of multiple omics approaches, will be crucial in overcoming these regulatory hurdles and enhancing vegetable improvement. Identifying and targeting specific genes responsible for desirable traits, particularly in complex genetic backgrounds, will be key to maximizing the benefits of genome editing.

Table 1: Application of CRISPR-Cas9-based editing of genes in vegetables.

Vegetable		Target gene	Modification/mutant trait	Delivery method	Reference
Fruit vegetables	Tomato	SlAGO7	KO/leaflets lacking petioles and later- formed leaves lacking laminae	*Agrobacterium*-mediated transformation	Brooks *et al.*, 2014 [16]
		RIN	KO/incomplete-ripening fruits	*Agrobacterium*-mediated transformation	Ito *et al.*, 2015 [37]
		DELLA, ETR1	Substitutions/marker gene-free plants harboring stable DNA substitutions	*Agrobacterium*-mediated transformation	Shimatani *et al.*, 2017 [73]
		SLMAPK3	KO/reduced drought tolerance	*Agrobacterium*-mediated transformation	Wang *et al.*, 2017 [92]
		PROCERA	KO/derepressed growth	*Agrobacterium*-mediated transformation	Tomlinson *et al.*, 2019 [82]
		SlCLV3	Mutation in *SlCLV3* promoter/ phenotypic changes in fruit size, flower morphology, and locule number	*Agrobacterium*-mediated transformation	Rodríguez-Leal *et al.*, 2017 [66]
		SP, SP5G, SlCLV3, SlWUS	Gene editing of coding sequences, *cis*- regulatory regions, or upstream open reading frames (ORF)/*de novo*-domesticated tomato	*Agrobacterium*-mediated transformation	Li *et al.*, 2018b [47]
		SP, O, FW2.2, CycB, FAS, MULT	Simultaneous CRISPR–Cas9 editing of six genes/modification of fruit number, size, shape, nutrient content, and plant architecture	*Agrobacterium*-mediated transformation	Zsögön *et al.*, 2018 [93]
	Cucumber	eIF4E	KO/resistance to ipomovirus, potyviruses zucchini yellow mosaic virus and papaya ring spot mosaic virus-W	*Agrobacterium*-mediated transformation	Chandrasekaran *et al.*, 2016 [19]
		CsWIP1	KO/gynoecious phenotype	Enhanced *Agrobacterium*-mediated transformation using vacuum infiltration	Hu *et al.*, 2017 [32]
	Watermelon	ClPDS	KO/albino phenotype	*Agrobacterium*-mediated transformation / PEG-mediated protoplast transfection	Tian *et al.*, 2017 [81]
		ClALS	Point mutation/herbicide resistance	*Agrobacterium*-mediated transformation	Tian *et al.*, 2018 [80]
		ClPSK1	KO/enhanced resistance to *Fusarium oxysporum* f. sp. *niveum*	*Agrobacterium*-mediated transformation	Zhang *et al.*, 2020b [88]
		ClWIP1	KO/gynoecious watermelon	*Agrobacterium*-mediated transformation	Zhang *et al.*, 2020a [88]
Leafy vegetables	Lettuce	LsBIN2	KO/targeted gene disruption in whole plants regenerated from protoplasts	PEG-mediated protoplast transfection	Woo *et al.*, 2015
		LsNCED4	KO/loss of thermoinhibition	*Agrobacterium*-mediated callus or somatic explants transformation	Bertier *et al.*, 2018 [84]
		LsGGP2	Deleted uORFs of LsGGP2 for increasing the translation of mRNAs/ increased oxidation stress tolerance and ascorbate content	*Agrobacterium*-mediated callus or somatic explants transformation	Zhang *et al.*, 2018a [89]
	Chicory	CiPDS	KO/albino phenotype	*Agrobacterium*-mediated transformation/ PEG-mediated protoplast transfection	Bernard *et al.*, 2019 [9]
	Chinese kale	BaPDS1, BaPDS2	KO or KD/albino phenotype	*Agrobacterium*-mediated transformation	Sun *et al.*, 2018 [78]
		BoaCRTISO	KD/yellow color of Chinese kale with improved market prospects	*Agrobacterium*-mediated transformation	Sun *et al.*, 2020 [77]
	Cabbage	BoPDS	KO/albino phenotype	*Agrobacterium*-mediated transformation	Ma *et al.*, 2019a [51]
				Electro-transfection in RNP delivery to protoplast	Lee *et al.*, 2020 [44]
		BoPDS1, BoSRK3, BoMS1	Multisite and multiple gene KO using an array of sgRNA-tRNA/male-sterile line	*Agrobacterium*-mediated hypocotyls transformation	Ma *et al.*, 2019b [51]

Conclusion

Advancements in genome editing technologies, such as base editing and prime editing, offer unprecedented precision in modifying genetic sequences, enabling the development of crops with improved traits. Techniques like CRISPR-Cas9 facilitate targeted modifications, enhancing resistance to pests, abiotic stresses, and boosting nutritional content across various vegetable species. Notable applications in crops like tomato, cucumber, and watermelon demonstrate the potential to improve traits and reduce undesirable characteristics. As research progresses, continued refinement of these tools and addressing regulatory challenges will be essential for unlocking their full potential in agricultural biotechnology and sustainable crop development.

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