

PROSPECTS FOR APPLYING CRISPR/Cas9 GENE EDITING AND ITS NEW-GENERATION TOOLS IN THE DEVELOPMENT OF TOMATO HYBRIDS FOR GREENHOUSE CULTIVATION

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Abstract

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown under greenhouse conditions worldwide. Achieving high and stable yields in a controlled environment requires hybrids that combine compact plant growth, pollination-independent fruit set (parthenocarpy), multiple disease resistance, long fruit shelf life and high nutritional quality. Combining such traits in a single cultivar by conventional breeding is time-consuming and labor-intensive. Over the past decade, CRISPR/Cas9 technology and its new-generation tools — base editing, prime editing, transcriptional regulation (CRISPRa/CRISPRi), and the Cas12a and Cas13 systems — have made targeted and precise modification of the tomato genome possible. This review analyzes the molecular basis of CRISPR technologies, the stages of their development, and their application to traits of strategic importance for greenhouse tomato production. Rapid domestication of wild species (de novo domestication), the role of genome editing in hybrid seed production, regulatory and biosafety issues, and the limitations of the technology are also discussed.

Keywords: CRISPR/Cas9, gene editing, tomato, greenhouse, parthenocarpy, hybrid breeding, base editing, prime editing, de novo domestication.



Introduction

Tomato is one of the leading vegetable crops worldwide in terms of nutritional and economic value, and a substantial share of it is grown in protected cultivation, i.e. in greenhouses. Although greenhouse production allows a stable year-round supply, the greenhouse microclimate (high humidity, limited pollination, dense planting in a confined area) imposes specific requirements that differ from those of open-field breeding.

A modern greenhouse cultivar or hybrid is expected to combine several traits simultaneously: a compact and manageable growth habit; stable fruit set that does not depend on pollinating insects; resistance to powdery mildew, tomato yellow leaf curl virus (TYLCV) and bacterial diseases; long shelf life and good transportability of the fruit; and high flavor and nutritional value. Combining these traits in a single genotype through conventional crossing and selection is a lengthy, labor-intensive process. Moreover, because most useful genes have been retained in wild species, their transfer into cultivated tomato is accompanied by undesirable traits (linkage drag).

After Jinek et al. revealed in 2012 that the CRISPR/Cas9 system functions as a programmable DNA endonuclease [1], the technology rapidly transformed plant breeding. Efficient editing in tomato was demonstrated in 2014 [2], and in subsequent years the precision and capabilities of the technology have steadily expanded (Figure 1). This review systematically analyzes the promising applications of CRISPR/Cas9 and its new-generation tools in greenhouse tomato production.

Molecular basis of the CRISPR/Cas9 system.

The CRISPR/Cas9 system originates from the adaptive immune system of prokaryotes and consists of two main components: a single guide RNA (sgRNA) and the Cas9 nuclease. The sgRNA recognizes the target DNA sequence through complementary base pairing; recognition requires a short PAM (protospacer adjacent motif; NGG for Cas9) sequence immediately downstream of the target. At the target site, Cas9 cleaves both DNA strands, creating a double-strand break (DSB).

The resulting break is repaired by the cell's natural DNA repair pathways. Non-homologous end joining (NHEJ) is error-prone: it introduces small insertions and deletions (indels) and usually results in loss of gene function (knockout). Homology-directed repair (HDR), in the presence of a donor DNA template, enables precise modifications such as gene replacement or the insertion of a defined allele; however, its efficiency in plant cells is low [3].

Genetic constructs are delivered into tomato mainly by *Agrobacterium tumefaciens*-mediated transformation. An important practical advantage is that, once the Cas9 and sgRNA genes have done their job (i.e. introduced the edit), they can be removed in subsequent generations by crossing or segregation. The result is an edited plant free of foreign DNA (transgene-free); in many countries, such plants are therefore subject to lighter regulation than GMOs [10].

New generations of gene editing tools. Although classical CRISPR/Cas9 is well suited for disrupting gene function, its capacity to introduce precise point modifications is limited. In recent years, a number of new tools have been developed to overcome this limitation (Table 1).

Base editing. Base editing enables precise substitution of individual nucleotides without creating a double-strand break in DNA. A Cas9 nickase (nCas9) with impaired catalytic activity is fused to a



deaminase enzyme: cytosine base editors (CBEs) perform C→T substitutions, whereas adenine base editors (ABEs) perform A→G substitutions [5, 13]. This approach is well suited for precisely reproducing point differences (SNPs) found in naturally occurring beneficial alleles or for introducing mutations that confer herbicide or disease resistance.

Prime editing. Prime editing is an even more versatile method based on the “search-and-replace” principle. In this system, nCas9 is fused to a reverse transcriptase, and an extended pegRNA both recognizes the target and encodes the desired edit [21]. The technology can introduce all types of point substitutions as well as small insertions and deletions without double-strand breaks or donor DNA. Its functionality has been confirmed in plants, including rice and wheat [24], and research on improving its efficiency in tomato is actively under way.

Transcriptional regulation (CRISPRa/CRISPRi). A catalytically dead Cas9 (dCas9) can be directed to a gene's promoter without cutting the DNA. When fused to activator domains, it enhances gene expression (CRISPRa); when fused to repressor domains, it suppresses expression (CRISPRi) [19]. This approach makes it possible to fine-tune quantitative traits (for example, the expression of genes involved in metabolite biosynthesis or stress responses) without altering the gene sequence.

Cas12a and multiplexing. Cas12a (Cpf1) is an alternative nuclease that recognizes a T-rich PAM (TTTV), generates staggered (sticky-end) breaks, and allows several genes to be edited simultaneously (multiplexing) using a single crRNA array [4]. This makes it an efficient tool for stacking several beneficial traits for greenhouse production at once (e.g. resistance + compactness + fruit quality).

RNA-targeting Cas13 and virus resistance. The Cas13 system recognizes and cleaves single-stranded RNA rather than DNA. This property is exploited for protection against RNA viruses and for controlling transcript levels [18]. Because viral diseases cause substantial damage to tomato (especially those that spread rapidly in greenhouses), this approach is regarded as a promising protection strategy.

Cis-regulatory (promoter) editing. Rodríguez-Leal et al. (2017) showed that editing promoter (cis-regulatory) regions, rather than the coding sequences of genes themselves, can generate a continuous range of quantitative trait variation [6]. For example, editing the promoters of genes that control flower and fruit development made it possible to fine-tune fruit size, inflorescence architecture and plant architecture. Unlike an “all-or-nothing” knockout, this approach allows the breeder to select precisely tailored variation.



Table 1 Comparative characteristics of CRISPR gene editing tools

System / generation	Mechanism of action	Main applications in breeding
CRISPR/Cas9	Double-strand DNA break upstream of a PAM (NGG) sequence; indels via NHEJ, precise insertion via HDR	Gene knockout, large deletions, gene (allele) replacement
Cas12a (Cpf1)	T-rich PAM (TTTV); staggered (sticky-end) break; multiplexing with a single crRNA array	Simultaneous editing of several genes; targeting AT-rich regions
Base editing	Cas9 nickase (impaired catalytic activity) + deaminase; C→T (CBE) or A→G (ABE) without DNA breaks	Precise introduction of point mutations; recreation of allelic SNPs
Prime editing	nCas9 + reverse transcriptase + pegRNA; no double-strand break and no donor DNA	Small insertions, deletions and all types of substitutions — “search-and-replace”
CRISPRa / CRISPRi	dCas9 (catalytically dead) + transcriptional activator/repressor; upregulation or downregulation of gene expression	Fine-tuning of expression levels without altering the gene sequence
Cas13 (RNA-targeting)	Recognizes and cleaves single-stranded RNA rather than DNA	Resistance to RNA viruses; control of transcript levels

Evolution of CRISPR genome editing technologies

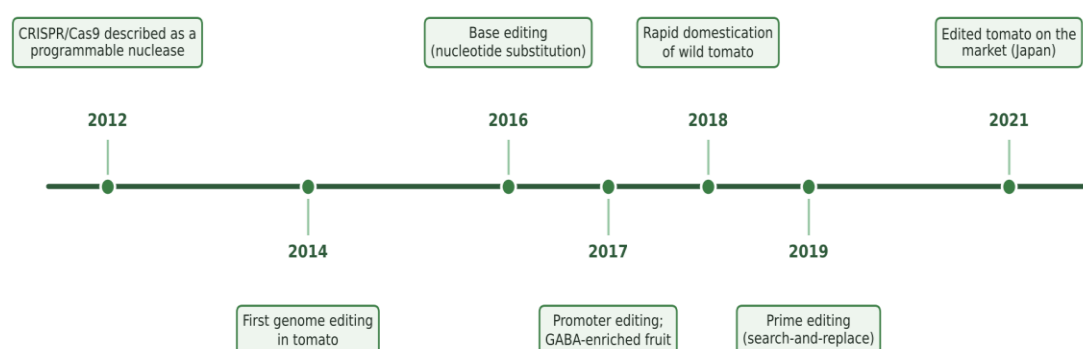


Figure 1. Evolution of CRISPR genome editing technologies (2012–2021).

Key traits for greenhouse tomato production and their editing

Growth habit, compactness and flowering control. For dense planting and suitability for mechanization in greenhouses, compact, determinate plants with uniform ripening are preferred. **SELF-PRUNING (SP)** and its homolog **SP5G** regulate growth habit, flowering time and day-length sensitivity. Knockout of SP5G confers day-neutrality and early, uniform yield [7]. Kwon et al. (2020) showed that editing the SP and SP5G genes can produce compact genotypes suitable for urban and vertical farming as well as for closed greenhouse systems [23].

Parthenocarpy — a strategic trait for greenhouses. Natural pollination is limited in closed greenhouses, and supplementary pollination by bumblebees or mechanical vibration is often required.

Parthenocarpy — fruit set without pollination and fertilization — solves this problem and ensures stable yields even under unfavorable conditions such as low temperature or high humidity. Knockout

of the *SlIAA9* gene results in parthenocarpic fruit set [8], whereas loss of *SlAGL6* function induces facultative parthenocarpy while retaining the ability to set normal seeded fruit [9]. This trait is one of the most practical achievements of CRISPR in greenhouse breeding.

Disease resistance. The humid greenhouse environment favors fungal and bacterial diseases. Deletion of the *SlMlo1* gene made it possible to obtain transgene-free tomato plants resistant to powdery mildew [10]. Loss of function of the *SIDMR6* ortholog confers broad-spectrum (multi-pathogen) resistance [25], while targeted editing of *SlJAZ2* confers resistance to bacterial speck [20]. Strategies against viral diseases based on CRISPR/Cas9 (targeting the TYLCV genome) [17] and Cas13-mediated RNA interference [18] are being developed. Such genetic resistance reduces the pesticide and fungicide load in greenhouses.

Fruit quality: shelf life, flavor and nutritional value. To improve tolerance to transport and storage, ripening-related genes — *RIN*, *ACS2*, pectate lyase (*PL*) and *lncRNA1459* — have been edited, resulting in delayed softening and extended shelf life [12, 14]. To enhance nutritional value, the gamma-aminobutyric acid (*GABA*) content was increased by editing the *SIGAD* genes [11]; a *GABA*-enriched tomato developed with this approach became the first CRISPR-edited food product to enter the market, in Japan in 2021 [26]. Such functional-food cultivars create high added value in the premium market segment.

Mechanical harvesting and fruit size. To simplify harvesting in greenhouses, jointless-pedicle genotypes are developed, and the *fw2.2*, *lc*, *fas* genes, which control fruit size and locule number, are edited according to the breeding objective. These genes play a decisive role in restoring fruit size, particularly during the domestication of wild species [15, 16] (Table 2).

Table 2 Major genes and traits targeted by editing in greenhouse tomato production

Target gene	Modified trait	Significance for greenhouse breeding
SP, SP5G	Determinate, compact growth; day-length insensitivity	Dense planting, early and uniform yield, suitability for vertical cultivation
<i>SlIAA9</i> , <i>SlAGL6</i>	Parthenocarpy (fruit set without pollination)	Stable fruit set in closed environments with limited pollinators
<i>SlMlo1</i>	Resistance to powdery mildew	Reduced fungicide load in humid greenhouses
<i>SIDMR6</i> , <i>SlJAZ2</i>	Broad-spectrum resistance and resistance to bacterial speck	Reduced need for chemical protection under multiple pathogen pressure
Coilin / TYLCV genes	Resistance to tomato yellow leaf curl virus (TYLCV)	Reduced damage from this whitefly-transmitted virus
<i>SIGAD2/3</i>	Increased gamma-aminobutyric acid (<i>GABA</i>) content	Functional-food (premium) product with high market value
<i>RIN</i> , <i>ACS2</i> , <i>PL</i> , <i>lncRNA1459</i>	Modified ripening and extended shelf life	Fruit tolerant to transport and long-term storage
<i>fw2.2</i> , <i>lc</i> , <i>fas</i> , <i>ENO</i>	Fruit size and locule number	Restoration of fruit size during domestication of wild species



Rapid domestication of wild species (de novo domestication). The cultivated tomato genome has narrow genetic diversity, and many beneficial alleles conferring stress and disease resistance have been retained in wild relatives. In the *de novo domestication* strategy, a resilient wild species (e.g. *Solanum pimpinellifolium*) is used as the starting material, and several genes that determine domestication traits — fruit size and shape, growth habit and ripening time — are edited simultaneously using CRISPR. Li et al. (2018) and Zsögön et al. (2018) showed that this approach can produce, within a single generation, tomatoes that combine both resilience and domestication traits [15, 16]. For greenhouse breeding, this is a powerful tool for rapidly developing promising cultivars without losing stress tolerance.

Role of gene editing in hybrid seed production. Greenhouse tomato production relies mainly on F₁ hybrids, as they provide heterosis, uniformity and trait superiority. A key issue in producing high-quality hybrid seed is preventing self-pollination in the maternal line; conventionally, this is achieved by manual emasculation (removal of the male floral organs), which is labor-intensive.

Gene editing can be used to create genetic male sterility by knocking out genes required for stamen development or pollen viability. This allows the maternal line to be used without emasculation and under precise control, simplifying hybrid seed production and improving seed purity. Editing self-compatibility/self-incompatibility genes is also a promising approach for managing crossing schemes.

Regulatory and biosafety issues. The legal status of gene-edited plants varies between countries. If an edited plant contains no foreign DNA (transgene) and the introduced change is indistinguishable from a natural mutation or from the outcome of conventional breeding, a number of countries (the USA, Japan, Argentina, Brazil and others) evaluate it separately from classical GMOs under a simplified procedure. It was on this basis that the GABA-enriched edited tomato was commercially launched in Japan [26]. At the same time, some jurisdictions (e.g. the European Union) apply a stricter regulatory approach.

In the context of Uzbekistan, compliance with national biosafety legislation and international practice, as well as ensuring consumer trust, are essential for introducing such technologies. Prioritizing transgene-free editing strategies offers a practical advantage in terms of regulation and public acceptance.

Limitations, challenges and prospects. Off-target effects: when the guide RNA is insufficiently specific, mutations may occur at unintended sites; this risk is reduced by designing highly specific sgRNAs and by verification through whole-genome sequencing.

- Regeneration difficulty: in some genotypes, regeneration of whole plants from cell and tissue culture is inefficient, which limits the scale of editing.
- Efficiency of HDR and prime editing: precise modifications (substitutions, insertions) are still relatively inefficient in plant cells, and the technology requires further improvement.
- Intellectual property and patent issues: some components of CRISPR technologies are patent-protected, so licensing must be taken into account in commercialization.





- Public acceptance: transparent information and science-based communication are essential to build consumer trust.

Nevertheless, the technology is advancing rapidly. Multiplexing, improvements in base and prime editing, and integration with high-throughput phenotyping and genomic selection are expanding the possibilities for the rapid development of multi-trait hybrids for greenhouse tomato production.

Conclusion

CRISPR/Cas9 and its new-generation tools have raised the efficiency of greenhouse tomato breeding to a qualitatively new level. Traits of strategic importance for greenhouse production, such as compact growth, parthenocarp, multiple disease resistance, long shelf life and high nutritional quality, can now be developed precisely and in a targeted manner. While base editing, prime editing, CRISPRa/CRISPRi and the Cas12a/Cas13 tools are expanding the precision and scope of editing, their application in de novo domestication and hybrid seed production is broadening the practical reach of the technology. Prioritizing transgene-free editing strategies and complying with national biosafety requirements are the key prerequisites for the promising and responsible introduction of these technologies in Uzbekistan.

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