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Genome Editing Technologies towards Tomato Improvement: Recent Advances and Future Perspectives

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ABSTRACT

Tomato (*Solanum lycopersicon* L.) is the world's second major vegetable crop and a superior model plant for studies on fruit biology. However, the changing climatic conditions are hugely impacting the yield and quality of tomato. CRISPR/Cas9 technology has been widely used in tomato breeding for enhanced disease resistance, herbicide tolerance, domestication and urban farming of wild tomato, and improved fruit yield and quality. Furthermore, new and advanced editing systems like Cas12a, Cas12b, base editing, and prime editing have been recently applied for high-precision tomato improvement. CRISPR variants, PAM-less genome editing, advanced transformation protocols, and gene delivery systems have played a critical role in fast breeding. This review offers an informative summary of recent progress in various genome editing methods and applications for improving tomatoes. It also focuses on critical issues, regulatory concerns, and prospects of genome editing platforms to improve tomato and allied crops.

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1 Introduction

Tomato (*Solanum lycopersicon* L.) is the second most crucial agronomic crop grown worldwide. With global production of more than 182 million metric tons in approximately 50 million hectares, tomato contributed to a trade value of 19.5 billion USD in 2018 (Khan et al. 2021). In addition to the bioactive compounds, it has significant physiological properties like anti-allergenic, antithrombotic, anti-inflammatory, antioxidant, antimicrobial, and vasodilatory (Kumar et al. 2022). However, many environmental factors highly affect tomato production, affecting global food security (Mishra et al. 2021). Over the last few decades, research has been focused on breeding toward tolerance to environmental stresses. It has resulted in identifying multiple stress-tolerant genes that can be used for tomato breeding programs (Gupta et al. 2022). Among the available tools, genetic engineering is the most important tool for crop improvement. Technological advances in functional genomics have made it possible to introduce multiple stress-responsive genes into plants and make them climate resilient. Although transgenic breeding has been overcome, most of the bottlenecks related to genetic recombination faced by traditional approaches, public concerns, and expensive regulatory processes continue to impede commercialization.

While the acceptance of gene-modified crops is debatable, genome editing techniques via sequence-specific nucleases (SSNs) have the potential to resolve these limitations. They could help develop multi-trait-modified crops. The SSNs cleave the double-stranded DNA at specific sites, which is healed through the cell's endogenous repair mechanism, resulting in a precise genetic mutation. Since 1988, when the first tobacco gene targeting experiment was conducted, multiple gene targeting tools, including the meganucleases, ZFNs (Zinc Finger Nucleases), TALENs (Transcription Activator Like Effector Nucleases), and the CRISPR system, have been developed and utilized in the improvement of multiple crop varieties (Ahmar et al. 2020). Among others, the CRISPR/Cas system has wide acceptance in the scientific community due to its simplicity, non-requirement of complex protein chemistry, and the ability for simultaneous introduction of double-stranded breaks (DSBs) at multiple sites (Mishra et al. 2019).

Since its implementation in 2014, gene modification has enormously aided precision breeding in tomato (Brooks et al. 2014). More insight into the intricacies of tomato fruit ripening genes has been reevaluated using targeted mutagenesis gene-editing machinery (Wang et al. 2019; Gao et al. 2020). CRISPR/Cas9 generated knockout mutations of numerous master regulators of tomato ripening have resulted in reduced ripening inhibition phenotype as compared to their naturally occurring mutations or RNAi lines, indicating that the fruit ripening regulation network is more intriguing than previous knowledge (Wang et al. 2021). Genome editing technologies significantly enhance fruit productivity and quality, stress resistance, and domestication (Xu et al. 2019; Kwon et al. 2020). Most recently, the base and prime editors have greatly improved the editing efficacy, expanding the scope for sustainable agricultural development (Mishra et al. 2019; Anzalone et al. 2020). In other words, CRISPR/Cas9 has revolutionized the basic research in plants for crop improvement. This review attempts to summarize the genome editing platforms and applications of CRISPR/Cas-based genome editing tools in tomato improvement, as well as the future perspectives of the editing approaches concerning basic research and crop improvement.

2 Advanced gene editing systems applied in tomato

Genome editing helps create a DNA mutation by deletions, insertions, or base substitutions in the target sequences. Such modifications can be performed using editing tools like TALENs, ZFNs, and CRISPR/Cas9 system (Wei and Li 2023). ZFNs and TALENs are first-generation genome editing tools that are costly, time-consuming, and difficult to design. In contrast, the CRISPR/Cas9 system is inexpensive, time-saving, and simple to build. Over time, several new Cas9 variants, such as SacCas9, NmCas9, and StCas9, have been discovered, which helped to improve target specificity and editing efficiency and decreased the off-target cleavage of this editing platform (D'Ambrosio et al. 2018).

2.1 CRISPR/Cas9

The discovery of CRISPR/Cas9 genome modification in 2012 has dramatically altered the field of plant science (Jinek et al. 2012). This tool is based upon the bacterial-acquired immune system acting upon exogenous invading genes or factors. These foreign gene fragments are retained as memory as spacer sequences comprising a CRISPR array (Koonin et al. 2017). The Cas protein and the spacer sequence are being used as surveillance systems to recognize and degrade foreign DNA or RNA. Adaptation, expression, and interference are three major steps in this process. Cas1 and Cas2 mediate the adaptation step to integrate foreign DNA fragments into the CRISPR locus of the host, followed by the production and maturation of transcribed gRNA by expression step. The last interference phase is completed by cleaving invaded DNA by Cas proteins, which are complex with mature gRNA (Monn et al. 2019).

The two essential elements of the CRISPR/Cas9 system necessary for genome modification are an adaptable single-stranded RNA (sgRNA) and the DNA endonuclease (Cas9) protein from *Streptococcus pyogenes* (Figure 1a). A sizeable globular recognition lobe and a tiny nuclease lobe make up the bilobed Cas9 protein with two nuclease domains, RuvC and HNH, and

these two domains are specific for each cut in a particular DNA strand. Effective editing requires a PAM (Protospacer adjacent motif) sequence close to the target location. For example, Cas9, derived from *S. pyogenes*, recognizes 5'-NGG-3' as PAM. Various types of Cas proteins have been discovered to overcome this limitation. Furthermore, Cas 12 identifies the sequence 5'-TTTN-3' or 5'-TTN-3' as a PAM. However, additional Cas9 variations (VQR, EQR, VRER) have been created to recognize different PAMs. As a result, there will be more opportunities to alter any target sequence in the genome (Asmamaw and Zawdie 2021).

2.2 CRISPR/Cas12a

CRISPR/Cas12a, formerly named/called CRISPR/Cpf1 (Figure1a), derived from *Prevotella* and *Francisella* 1, is one of the advanced forms of CRISPR used in plant gene modification (Endo et al. 2016). A significant difference between CRISPR/Cas9 and CRISPR/Cas12a is its location and reorganization sequence of PAM. Various monocot and dicot plants have been modified/improved using CRISPR/Cas12a. CRISPR/Cas9 needs a PAM sequence that is rich in G at the 3' end of the target sequence (5'-NGG-3'), whereas in the case of CRISPR/Cas12a, it targets the T-rich PAM sequence (5'-TTTN-3' or 5'-TTN-3') at the 5'end which results in high efficiency for cleavage (Zetsche et al. 2015). CRISPR/Cas12a functions without a trace RNA for cleavage. The complex of Cas12a-crRNA can cleave the targeted DNA efficiently. The crRNA containing the repeat of 40-45 nucleotides long along with the spacer can edit the genome more effectively when compared with the sgRNA of CRISPR/Cas9, which contains nearly 100 nucleotides (Zetsche et al. 2015). Cas12a includes dual enzymatic activity like RNAase and nuclease. The RNAase activity helps process pre-crRNA into crRNA, and the nuclease activity helps cleavage dsDNA. CRISPR/Cas12a can generate multiple crRNAs by the involvement of a single promoter, which makes it simpler when compared with CRISPR/Cas9. The off-target cleavage activity of Cas12a is also low compared to CRISPR/Cas9. Furthermore, the RuvC and Nuc domains cut the targeted sequence at the 25th base and the non-targeted sequence at the 17th base, resulting in cohesive ends with five base-pair overhangs (Zetsche et al. 2015). Genome editing in the tomato geminiviral replicon using the CRISPR/Cpf1 system was up to three times more effective than the CRISPR/Cas9 system. A single replicon system by CRISPR/Cas9 was converted into a multi-replicon system using CRISPR/LbCas12a-based HDR (Vu et al. 2020). Three orthologs of Cas12a, namely, the *AsCas12a* from *Acidaminococcus* sp.BV3L6, *FnCas12a* from *Francisella tularensis* subs *pnovicida* U112, and *LbCas12a* from *lachnospiraceae* bacterium ND2006 are currently in use for precise modification in Tobacco, Arabidopsis, tomato, cotton, and rice (Endo et al. 2016; Kim et al. 2017).

2.3 Base editing

The base editing system includes two DNA base editors, i.e., adenine base editors (ABEs) and cytosine base editors (CBEs); these two do not require a double-stranded break or a donor template for an immediate and irreversible change of one targeted base pair into another (Komor et al. 2016; Nishida et al. 2016). These base editors are created by joining a dormant Cas9 domain, cytosine deaminase domain, and an inhibitor of uracil glycosylase (Eid et al. 2018) (Figure 1b). Base editors recognize a specific NGG PAM sequence and function only when the base editing window adheres to the target region (Komor et al. 2016; Gaudelli et al. 2017). The utilization of specific PAM sequences lowers editing efficiency in this system. To overcome this issue, several base editors with PAM flexibility have been engineered, and novel ABEs and CBEs have been developed by using variants of Cas9 with recognition of different PAM sequences other than NGG (Endo et al. 2019; Qin et al. 2019). These enhanced base editors offer the opportunity to boost base editing's effectiveness and broaden its use by targeting different plants. The SpCas9 variants like EQR-BE3, SaKKH-BE3, VQR-BE3, and VRER-BE3 with target NGCG, NRT, NGAN, and NGCG PAMs, respectively, increased the efficiency by about 2.5 folds (Kim et al. 2017).

CBEs help in the conversion of Cytosine-Guanine (C-G) base pairs into Thymine-Adenine (T-A) base pairs (Li et al. 2017; Lu et al. 2017). In 2017, the CBEs were first applied in tomato to edit two hormone-signaling genes, i.e., ETR1 and DELLA, showing a base editing efficiency of 26.2% to 53.8% (Shimatani et al. 2017). Target-AID base editing technology targeted three genes related to carotenoid accumulation: SlDDB1, SlCYC, and SlDET1. It was observed that allelic variation occurs due to base substitution from cytidine to thymine, which results in the difference in the accumulation of carotenoid content (Hunziker et al. 2020). The branched-chain amino acid biosynthesis pathway includes the acetolactate synthase (ALS) genes. The mutation of the *ALS1* gene in the tomato plant successfully created resistance towards chlorsulfuron, and 12.9% of these plants were transgene-free (Veillet et al. 2019). In the tomato plant, the *ALS* genes were also involved in causing mutation of proline 186 residue, and the base editing efficiency of *ALS*1 pro-186-residue codon was up to 71.4 %. Three additional tomato genes, viz., mDNA Damage UV Binding Protein 1, Deetiolated 1, and Lycopene beta cyclase, were subjected to CBE-mediated nucleotide substitutions, and the results demonstrated a considerable rise in total lycopene, carotenoid, and carotene level (Hunziker et al. 2020).

2.4 Prime editing

To improve precision in genome modification, prime editing is the most recently developed tool implemented for specific mutations in crop plants (Anzalone et al. 2019). The prime editing system

comprises three components, including the prime editing guide RNA (pegRNA), an engineered Moloney murine leukemia virus (M-MuLV) reverse transcriptase, which fused with the C terminal end of Cas9 (H840A), and a nickase that helps to avoid double-stranded break formation (Scholefield and Harrison 2021) (Figure 1c). Cas9 nickase cuts in the non-complimentary strand of DNA just after three nucleotides upstream to the PAM site. After the cut, a DNA flap is exposed, generating a 3'OH group that binds with the primer binding site (PBS) of the RNA template and serves as a primer for the reverse transcriptase enzyme, which is responsible for extending the 3' flap by copying the edited sequence of pegRNA. Comparatively to the unedited 5' flap, it is less preferable to hybridize with the unedited complementary strand after extending the 3' flap. The endonuclease FEN1 helps in the excision of the 5' flap, which favors the hybridization of the edited 3' flap (Scholefield and Harrison et al. 2021). The PE system has been tested in several crop species, such as rice (Butt et al. 2020; Xu et al. 2020a; Li et al. 2020), tomato (Lu et al. 2021), maize (Hua et al. 2020; Jiang et al. 2020), wheat (Lin et al. 2020; 2021; Li et al. 2022a) and potato with promising results (Perroud et al. 2022).

Parameters that define the effectiveness of prime editing include thermostability, source of reverse transcriptase enzyme, primer binding site sequence length, length of reverse transcriptase template, and nicking sgRNA in unmodified strand (Lin et al. 2020). Mutations that make reverse transcriptase more thermostable and capable of attaching to its target location also increase the editing efficiency up to 3-fold (Anzalone et al. 2019). Different RT sources show varying efficiency in editing, such as the Cauliflower Mosaic Virus (CaMV) derived RT demonstrated low editing compared to that from Molony Murine Leukemia Virus (MMuLV). Furthermore, the efficiency of prime editors was strongly affected by the length of the RT template but not significantly by the primer binding site's size and the sgRNA nicking site (Anzalone et al. 2019). The pegRNA secondary structure and the G/C composition of the primer binding site may impact the effectiveness of prime editing. Prime editors have low off-target editing frequency compared to the CRISPR/Cas9 system. Its hybridization occurs between the spacer region of pegRNA and the target DNA, the primer binding site of pegRNA, and the edited DNA flap (Marzec et al. 2020). In prime editors, the efficiency of mutation types varies. It was reported recently that the deletion frequency of 6 bp can be up to 21.8%, insertion of 3 bp frequency can be up to 19.8%, and the frequency of point mutation can be up to 0.03-18.75% in rice (Xu et al. 2020b; Lin et al. 2020). In the case of plants, there is also a decrease in the indels with the increase of targeted deletions or insertion (Lin et al.2020).

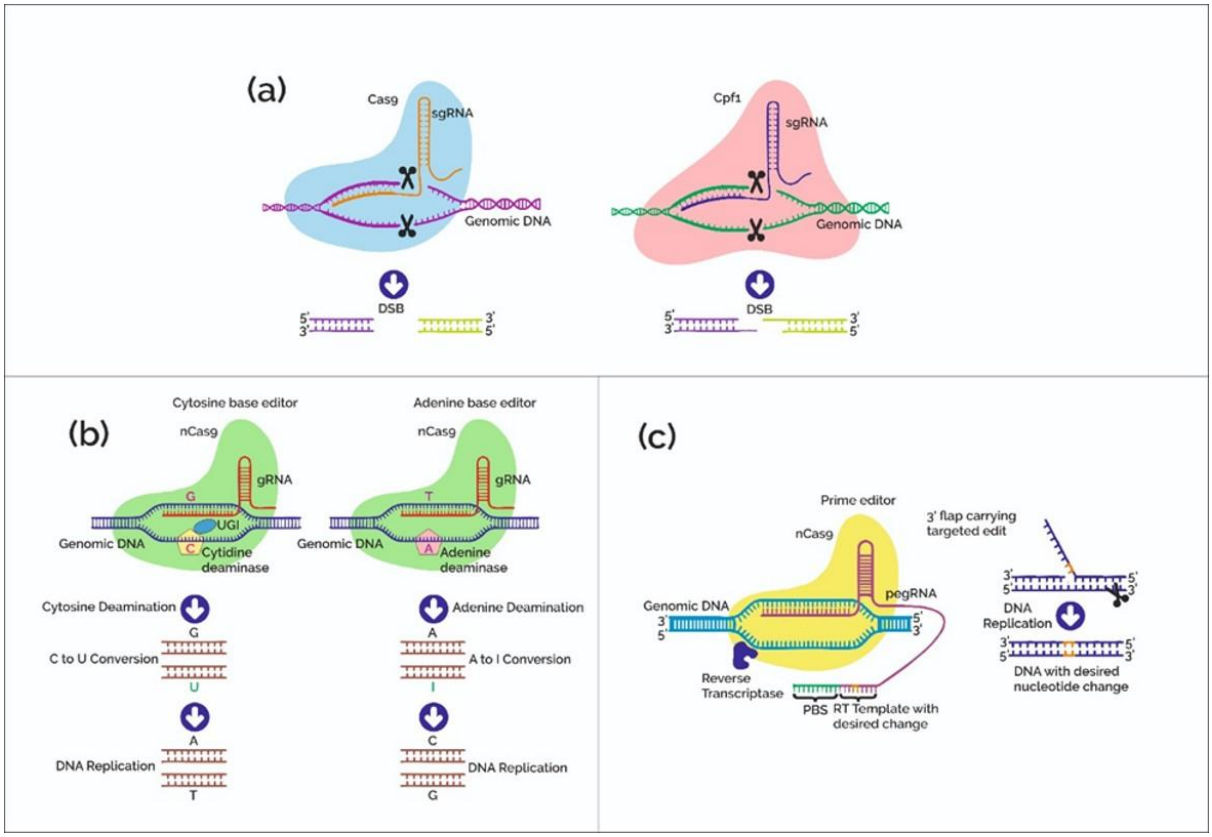

Figure 1a: A schematic diagram showing CRISPR/Cas9 and Cas12 (Cpf1) genome editing system, 1b: Showing the schematic diagram for cytosine base editing and adenine base editing, and 1c: is the schematic diagram for Prime editing

In plants, prime editing adequacy is usually low compared to human cells. All the prime editors in plants so far reported using RNA polymerase III promoters like U6 promoter for the expression of pegRNA. Previous studies in CRISPR/Cas9 systems use RNA polymerase II promoters such as CmYLcv to improve editing efficiency up to 2 folds in plants (Li et al. 2022b). So, an alternative RNA polymerase like CmYLcv can be used to improve the efficiency of prime editing, or for the expression of pegRNA U3 promoter can be used. In another study, by combining different optimization techniques, such as changing the PE design to PEmax and expressing engineered pegRNA with a structured motif under the direction of a composite promoter, the plant prime editor 2 (enpPE2) was created (Li et al. 2022c). The resultant T_0 rice plants exhibited higher editing frequencies (64.58% to 77.08%) than the unmodified pPE2. The study indicates that the enpPE2 system can be a solid and effective technique for altering plant genomes precisely.

3 Impact of CRISPR/Cas9-based genome editing on plant productivity and stress tolerance

CRISPR/Cas9 Genome editing is a highly efficient tool for improving tomato varieties concerning biotic and abiotic stresses, high yield potential, and enhanced shelf life (Figure 2). Furthermore, the technology has effectively met demands by improving fruit quality and yield (Ito et al. 2015; Ueta et al. 2017).

3.1 Yield and quality improvement

Tomatoes are an important agronomic crop, and their productivity is determined by flowering speed, flowering number, size, and fruit number. CRISPR/Cas9 induced mutation in the promoter of *CLV3* signaling peptide, alteration in *COMPOUND INFLORESCENCE (S)* gene governing inflorescence architecture known as *SELF PRUNING (SP),* and cis-regulatory frameshift through CRISPR/Cas9 has enhanced the number and size of the floral organs in the fruit, resulting in a boost in tomato yield (Rodriguez-Leal et al. 2017). Thus, CRISPR/Cas9-induced mutations in the promoter region can be exploited to develop a new cultivar with changes in quantitative traits to boost agricultural yield. Color, shape, size, nutrition, sweetness, scent, acidity, and shelf life are all fruit-quality components. Consumers increasingly demand higher fruit quality, and genome engineering has been effectively utilized to increase tomato fruit quality genetically. Fruit color is due to the accumulation of pigments such as carotenoids and flavonoids and the degradation of chlorophyll content during the ripening of fruits. Since red-colored tomatoes are more prevalent, other hues of tomatoes are also in demand in the market for different consumer groups. Furthermore, mutation of multiple genes like *PSY1, SGR1, and MYB12* produced green tomatoes by only affecting the biosynthesis or accumulation of pigments without affecting the yield and fruit quality (Yang et al. 2023). Knockout of *slsp/sler and slsp5g* multiplex mutation increased the compactness and yield of tomato plants (Kwon et al. 2020). Likewise, a group of five genes in the carotenoid metabolism pathway of tomatoes has been engineered using a multiplex CRISPR/Cas9 system (Li et al. 2018b). Surprisingly, multiplexed tomato fruit shows an increase in lycopene concentration of 5.1 times. Total carotenoid, lycopene, and carotene levels also dramatically increased due to CBE-mediated sequence alterations in three more tomato genes implicated in the accumulation of carotenoids (Hunziker et al. 2020). *NAC* transcriptional factors play a critical role in fruit ripening, and *SNAC9* is involved in various metabolic pathways of ethylene and abscisic acid. CRISPR/Cas9 mutation of *SNAC9* affected the carotenoid metabolism, which decreases carotenoid

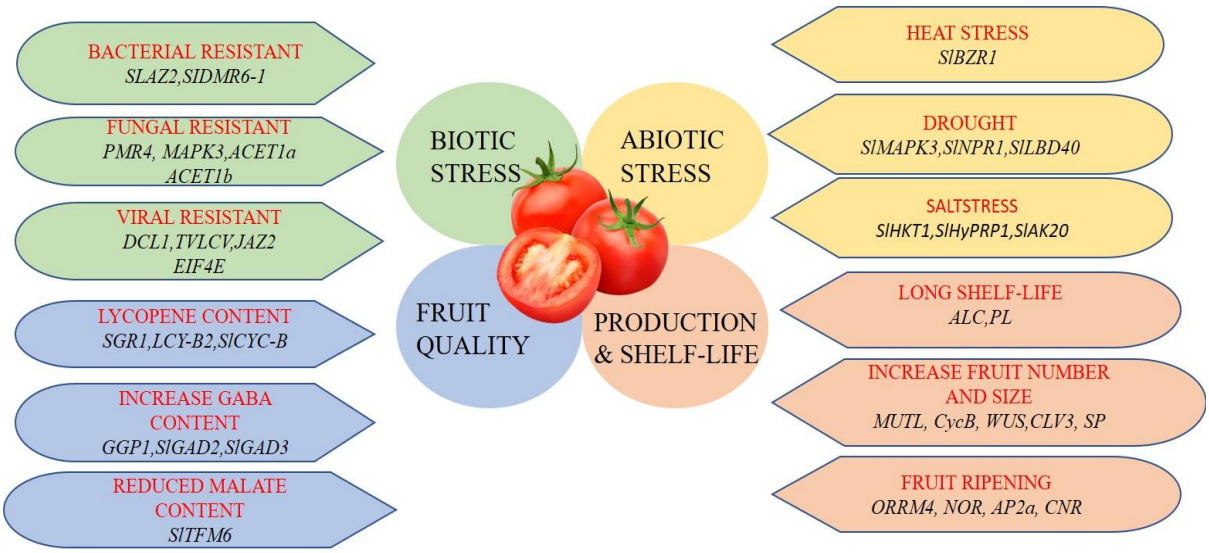

Figure 2 CRISPR/Cas9 genome editing of genes involved in tomato improvement.

and lycopene content as well as degradation of chlorophyll content and mutant *SNAC9* delayed fruit ripening by altering the gene expression levels responsible for metabolism of pigment *PSY1, PDA, CRTISO, LCYE, Z-ISO, SGR1, DXS2, LCYB* and *CrtR-b2* genes (Feng et al. 2023).

3.2 Improving nutritional value

Tomato fruit has a high concentration of gamma-aminobutyric acid (*GABA*), a human functional component that acts as an inhibitory neurotransmitter (Bachtiar et al. 2015). Knockout of *GABA-TP1*, *GABA-TP3*, *CAT9*, and *SSADH* increased the *GABA* content by 19 folds in tomato with high oxalic acid content, which has anti-nutrient metabolite (Li et al. 2018b). It has been proven that vitamin D3 is more beneficial than vitamin D2, and vitamin D3 is present in tomato leaves but not in fruits. It is stored as an intermediate in the form of SGAs in the fruits. Multiplex-editing of five genes, including three GABA transaminase genes, a cationic amino acid transporter *(CAT9)*, and a succinate semialdehyde dehydrogenase (*SSADH)* gene, led to a significant increase in the GABA content of tomato (Figure 3) (Li et al. 2018a). A key enzyme in the synthesis of GABA is glutamate decarboxylase (GAD), and induced mutations in GAD genes *SlGAD2* and *SlGAD3* in tomato increased GABA buildup in tomato fruit by 7 to 15-fold (Nonaka et al. 2017).

3.3 Production of seedless tomato

Seedless fruit production without fertilization is an important trait that confers various benefits in agriculture. When compared to the wild-variety plant, mutations in the auxin/indole-3-acetic acid (Aux/IAA) producing gene *SlIAA9* and two *AUXIN RESPONSE FACTORS (ARFs)-SlARF7* and *SlARF5*- led to seedless tomato and morphological alterations in leaves (Ueta et al. 2017). *SLAGL6* regulates the change from the ovarian arrest state that prevents anthesis to the fertilization-triggered fruit set. Parthenocarpic fruit, a fertilization-independent fruit, can be considered a valuable goal as problems are faced during fertilization due to global warming. Even in heat stress, CRISPR/Cas9 mutation of *SlAGL6* led to the formation of parthenocarpic fruit (Klap et al. 2017).

3.4 Genome editing for fruit ripening/fruit quality

Fruit ripening is a complex biological process that involves multiple biochemical, physiological, organoleptic, and metabolic changes. Ripening of fruit causes sugar accumulation, softening of fruits, color and volatile chemical accumulation, and a decrease in organic acid concentration. Fruit quality and quantity are the two main important factors for crop improvement. Tomato is one of the model plants for fruit biology research, and CRISPR/Cas9 knockout technology has been effectively used to study various

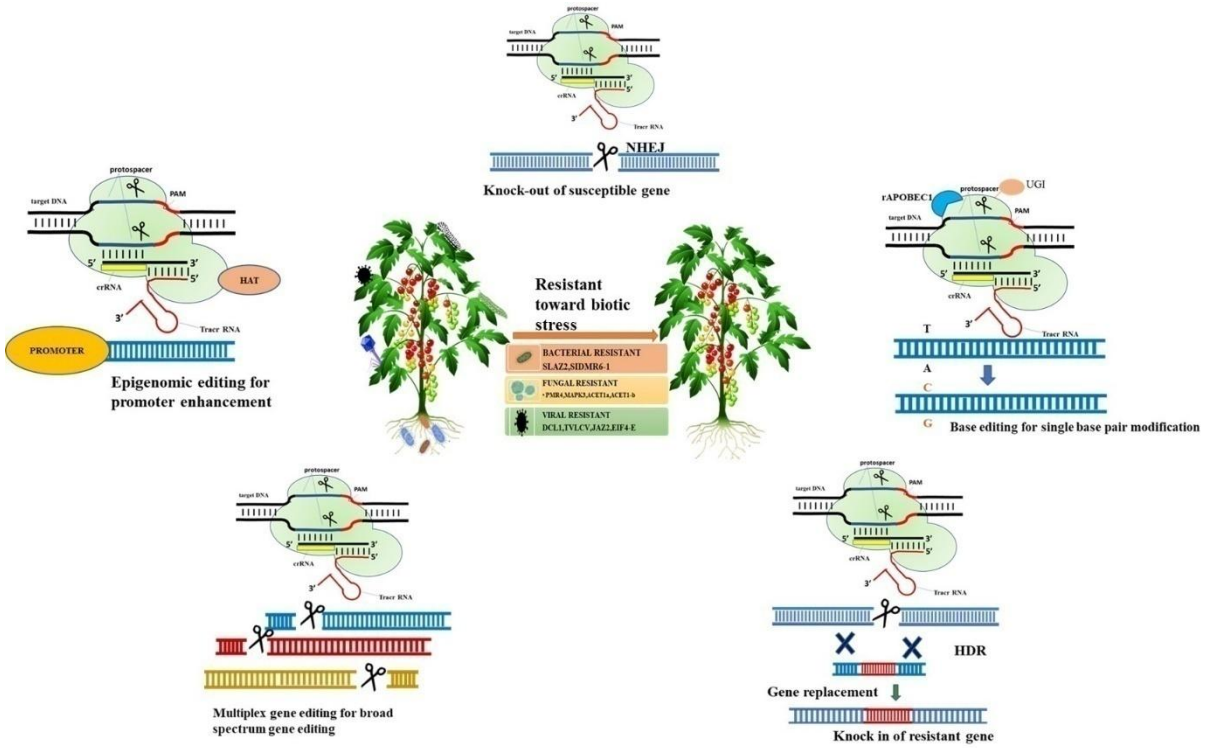

Figure 3 Strategies of genome editing towards plant tolerance to multiple diseases and crop improvement by knocking out susceptible genes through epigenomic editing for promoter enhancement, multiplex genome editing for targeting numerous genes, gene replacement by homologous direct repair, base editing for particular base change and repair by non homologous end joining method.

functions regulated by transcriptional factors and signaling pathways for architecture, growth, development, ripening of fresh fruits, and for the shelf life of tomato (Wan et al. 2021). Since 2014, genome editing has been used for precision engineering in tomato (Brooks et al. 2014) and revisiting the earlier work performed for fruit ripening by RNA silencing. It was noticed that CRISPR/Cas 9 editing system shows weaker ripening inhibitory symptoms when compared with wild-type or RNAi plants (Wang et al. 2020). CRISPR/Cas9 mediated modification of the *RIPENING INHIBITOR (RIN)* gene has contributed to the suppression of fruit ripening in tomatoes (Xu et al. 2020b) (Figure 3). Knock out the polygalacturonase gene in tomato *(SlPG),* which is responsible for fruit firmness, and the mutant *slug* delayed the softening of tomato fruit (Nie et al. 2022). The sweetness of tomatoes is essential for providing a saucy taste due to the amount of fructose, glucose, and TSS content. Gene editing of specific genes like *SlINVINH1* and *SlVPE5* improves the sweetness by increasing the amount of glucose, fructose, and TSS (Wang et al. 2021). Ascorbate is one of the essential products of tomatoes and is used as a supplement for the human diet. A negative regulator of the producing gene *SlAPX4* was knockout, resulting in high ascorbate production in ripened tomatoes (Do et al. 2022). Mutation of *SlAS2* or *SlAS2L* decreased the thickness of the pericarp by reducing the cell layer and cell area, which decreases fruit size. Stamens and leaves also exhibited several morphological defects in single and double mutants (Dong et al. 2023).

3.5 Targeting photoperiodic response

Flowering at the correct time is critical not just for reproductive potential but also for yield optimization. Different genes are responsible for regulating tomato flowering in terms of day length. *SP5G*, a Blooming LOCUS T-like gene, acts as a flower repressor, controlling the flowering state for the day. SELF-PRUNING GENE (*SP gene*) is an Arabidopsis *(TFL1)* TERMINAL FLOWER 1 ortholog that encodes for a flowering repressor gene in Tomato. Double mutations on *sp5g sp* resulted in early fruit ripening and flowering bursts (Soyk et al. 2017). Knockout of *SP5G* shows quick flowering and increased compact and determinate growth of tomato (Soyk et al. 2017). Domestication of tomato has been done by using CRISPR/Cas knock out of specific genes like the Self-pruning *SP* gene for determinate plant growth, *FAS MULT, and FW2.2* gene for increasing size, number, and weight of the fruit (Agustin et al. 2018).

A knockout mutation of *HEL* via CRISPR/Cas9 generated vine-like fruits (Yang et al. 2020). In contrast, *CRABS CLAW(CRC*) orthologues are crucial in determining floral meristem and gynoecium formation across angiosperms, which help perfect flower and fruit formation. So, for a clear understanding of the mechanism of CRC-mediated flower meristem regulation, CRISPR/Cas9 mediated knock out of *SlCRCa* was performed, and indeterminate floral meristem was formed, and it was clear that CRC mediates the floral meristem termination (Castaneda et al. 2022). *SlDOF9* negatively modulates the floral differentiation in tomatoes, and the knockout of *SlDOF9* significantly influences the differentiation of inflorescence and floral meristem (Hu et al. 2022). Additionally, the knockout of a set of kinase-inducible domain interacting genes, i.e., *SlKIX8* and *SlKIX9*, resulted in enlarged fruit with increased pericarp due to cell expansion and dome-shaped leaves (Swinnen et al. 2022). B-box transcription factor *BBXs* plays a major role in the development of plants, and SlBBX4 in tomatoes has a role in photomorphogenesis. Mutation of *slbbx4* resulted in a hypersensitive response towards red light and a normal response towards far-red, blue, and UV-B light in hypocotyl length assays. Mutant *slbbx4* under long and short-day conditions delayed flowering (Xu et al. 2023).

3.6 Engineering biotic stress resistance in tomato

Plants are continually assaulted with biotic and abiotic stresses, and various genes are associated with plant stress responses. Tomato breeders' primary goal is always to improve tomatoes' tolerance to environmental stress (Tieman et al. 2017).

3.6.1 Development of bacterial-resistant tomato

The bacterial pathogen *Pseudomonas syringae* causes a broad range of diseases in tomatoes. So, it was a great challenge to solve this problem. *P.syringae* Pv.tomato DC3000 (PtoDC3000), the causal agent of bacterial speck disease, helps the pathogen produce coronatine, which stimulates stomata opening and facilitates the colonization of bacteria in the leaf. In *Arabidopsis*, *AtJAZ2* acts as a co-repressor for COR, and the dominant mutation of *AtJAZ2* Jas repressor is resistant to proteasomal degradation and prevents the opening of stomata by COR. In tomato, an orthologue of AtJAZ2 was identified named *SlJAZ2*, and CRISPR/Cas9 mutation of *SlJAZ2* (Figure 3) was used for generating JAZ2 repressor, which lacks the C terminal of JAS and prevents reopening of stomata. This resulted in the resistance of tomatoes to the pathogen, which causes bacterial specks. Besides this, grey mold disease remained unaltered in mutated *SlJAZ2* plants and helped in broad-spectrum resistance in tomatoes (Ortigosa et al. 2019). *Clavibacter michiganensis(Cm)* causes bacterial cancer, one of the most destructive diseases in tomatoes. *SlWAT1* showed susceptibility in tomato, and knockout of *SlWAT1* in tomato decreased the free auxin and ethylene synthesis in the stem of tomato, suppressing the expression of bacterial virulence factor (Koseoglou et al. 2023).

3.6.2 Tweaking CRISPR/Cas9 to enhance fungal resistance in tomato

The fungal pathogen has an excessive impact on agricultural fields, which causes various diseases like Smut, mildew, etc. Powdery

mildew is a calamitous fungal disease caused by the obligate biotrophic fungus *Oidium neolycopersici* that affects tomatoes worldwide. Susceptible genes are the main factors that cause disease by responding to pathogens. *MILDEW-RESISTANT LOCUS O* (*Mlo*) is a conserved S gene found in monocots and dicots that confers susceptibility to powdery mildew fungus (Acevedo-Garcia et al. 2014). Knockdown of *SlMlo1* reduced susceptibility to powdery mildew (Nekrasov et al. 2017). Powdery Mildew Resistant 1 (*PMR1* to *PMR4)* are four *S*genes in *Arabidopsis* that confer susceptibility to the fungus that causes powdery mildew. The orthologue of *PMR4* has been identified in tomatoes, and CRISPR/Cas9 modification of *SlPMR4* enhances disease resistance against the powdery mildew pathogen *O. neolycopersici* (Santillán Martínez et al. 2020). Another susceptible gene, *DMR6*, belongs to the 2-ODDs 2-oxoglutarate Fe(II)-dependent dioxygenases and is upregulated during pathogenic infection in *Arabidopsis thaliana*. Orthologous *DMR6* has been found in tomato *SlDMR6-1 and SlDMR6-2*, of which *SlDMR6-1* increased the SA level during pathogen infection, and *SlDMR6-2* helped balance SA levels in fruits and flowers. (Table 1, Figure 3) Knockout of *SlDMR6-1* shows resistance toward bacterial spots (Thomazella et al. 2021). *Fusarium* wilt caused by *Fusarium oxysporum f. sp. lycopersici (Fol)* is one of the destructive diseases. *XSP10 (Xylem sap protein 10) and SlSAMT* (*Salicyclic acid methyl transferase*) are the two susceptible genes for fusarium wilt (Debbarma et al. 2021). CRISPR/Cas9 mutation of *XSP10* and *SlSAMT* showed high tolerance towards fusarium wilt when dual editing was performed compared to single gene mutation (Debbarma et al. 2023).

Table 1 List of *Solanum lycopersicum* genes targeted for genome modification via CRISPR/Cas9 genome editing

Traits	Target genes	Target gene function	Editing Strategy	Genetic effects	References
Heat stress	*SlBZR1*	developmental process and stress response	CRISPR/Cas9	heat stress tolerance	Yin et al. 2018
	SlHyPRP1	Involved negatively in multi-stress responses	CRISPR/Cas9	Multi-stress is tolerant like stress tolerant, high quality, high yield, and susceptible to fusarium wilt	Tran et al. 2021
Drought stress	*SlMAPK3*	*MAPKs* (Mitogen-activated protein kinases) are the signaling molecule that responds to drought stress.	CRISPR/Cas9	Induced through drought stress	Wang et al. 2017
	SlNPR1	*NPR1* is the regulator of plant defense mechanism towards pathogens and SlNPR1 response towards both biotic as well as abiotic stress	CRISPR/Cas9	Mutant plant shows reduced drought tolerance	Li et al. 2019
	SlLBD40	*LBD40* is involved in jasmonic acid signaling and acts as a negative regulator of drought tolerant.	CRISPR/Cas9	Boost drought tolerant	Liu et al. 2020a
Cold stress	*SlCBF1*	*CBFs* are highly conserved C-repeat binding factors are the cold response components.	CRISPR/Cas9	Reduced chilling tolerance	Li et al. 2018a
Salt stress	*SlHKT1, 2*	*SlHKT1;2* is a salt tolerant allele.	LbCpf1	Targeted salt-tolerant gene1;2 and was tolerant towards salt tolerance	Vu et al. 2020
	SlHyPRP1	*HyPRP1* is a negative regulator for salt stress.	CRISPR/Cas9	Salinity tolerance	Tran et al. 2021
Herbicide	*SlALS1*, *SlALS2*	*ALS* is the key enzyme for the biosynthesis of amino acids like leucine, isoleucine, and valine, and also it targets commercial herbicides.	CBE	Herbicide-resistant	Veillet et al. 2019
Bacterial	*SlJAZ2*	*JAZ2* is expressed constitutively on stomata and hijacked by COR, which is produced by bacteria that help in the suppression of SA-dependent closure of stomata and promote penetration of bacteria	CRISPR/Cas9	Bacterial speck resistant	Ortigosa et al. 2019
	SlDMR6-1	*DMR 6-1* gene is upregulated during pathogen infection.	CRISPR/Cas9	Resistance towards bacterial pathogens like *Xanthomonas spp, P. syringes, P. capsicum,* and different oomycete	Thomazella et al. 2021

Traits	Target genes	Target gene function	Editing Strategy	Genetic effects	References
Fungal	*SlMPR4*	*MPR4* negatively regulates biotic stress	CRISPR/Cas9	Resistance towards powdery mildew fungus *Oidium neolycopersi*	Santillán Martínez et al. 2020
	SlMYC2	*MYC2* is a transcription factor and a main regulator of the MeJA signaling pathway	CRISPR/Cas9	Decrease in disease resistance towards *B. cinerea*	Shu et al. 2020
Viral	*DCL2b*	*DCL2b* is a key player in the biogenesis of small RNA and antiviral defense	CRISPR/Cas9	Susceptibility towards Tobacco mosaic virus (TMV), Potato virus X, Tobacco mosaic virus (ToMV)	Wang et al. 2018
	JAZ2	*JAZ2* is expressed constitutively on stomata and hijacked by COR, which is produced by bacteria that help in the suppression of SA-dependent closure of stomata and promote penetration of bacteria	CRISPR/Cas9	Resistance against banana streak virus	Ortigosa et al. 2019
	EIF4E1	*eIF4E* and its isoforms are the recessive resistance gene for potyviruses	CRISPR/Cas9	Resistance towards potyvirus PepMoV	Yoon et al. 2020
	slosca4.1	*OSCA4.1* are the key plant drought resistance regulators involved in pathogen infection. SlOSC4.1 contributes to the proper regulation of calcium homeostasis, which is required for PepMV infection	CRISPR/Cas9	Resistance towards PePMV	Ruiz-Ramon et al. 2023
Weed	*More Axillary Growth1*	*MAX1*are involved in the synthesis of strigolactones, which are the inhibitors of branching that are needed for germination of root parasitic weed	CRISPR/Cas9	Resistance towards root parasitic weed *Pheliancheaegyotiaca*	Bari et al. 2021a
Harvest quality	*Rin*	*RIN* has an important role in fruit ripening	NHEJ	Incomplete ripening of fruit	Ito et al. 2015
	Self-pruning 5G(SlSP5G)	*SP5G* is involved in photoperiodic sensitivity in plants	CRISPR/Cas9	Day-length-sensitive flowering	Soyk et al. 2017
	CRTISO and PSY1	*CRTISO and PSY1* genes are involved in the biosynthesis of the carotenoid pathway	CRISPR/Cas9	Yellow and orange tomatoes	Dahan-Meir et al. 2018
	SlPSY1(Phytoene synthase1)	*PSY1* genes are important genes of carotenoid biosynthesis	CRISPR/Cas9	Yellow fresh tomatoes	D'Ambrosio et al. 2018
	MYB12	*SlMYB*is a transcription factor that is involved mainly in the flavonol biosynthesis branch	CRISPR/Cas9	Pink tomatoes	Deng et al. 2018
	SlGAD2 and SlGAD3	*SlGAD2 and SlGAD3* are the key enzymes in the biosynthesis of GAB, A C terminal auto-inhibitory domain is present, which regulates enzymatic function, and removing this domain regulates enzymatic function.	CRISPR/Cas9	Increase of GABA accumulation from sevenfold to 15-fold	Nonaka et al. 2017
	NOR	*NOR* gene is mostly involved in fruit ripening	CRISPR/Cas9	The ripening process was partially affected	Wang et al. 2019
	AP2a	*SlAPA2a* acts as a negative regulator of fruit ripening.	CRISPR/Cas9	Initiated ripening of fruit in early stage but did not ripen fully	Wang et al. 2019
	FUL1/FUL2	*FUL1 and FUL2* are the transcription factors of the MADS-domain and are involved in regulating flowering time, fruit ripening, and architecture of inflorescence	CRISPR/Cas9	Double mutants did not show a ripe red color	Wang et al. 2019

Traits	Target genes	Target gene function	Editing Strategy	Genetic effects	References
	BZR1	*BZR1* is the regulator of brassinosteroid and is involved in developmental as well as stress tolerance	Loss of function	Decrease in heat stress tolerance	Yin et al. 2018
	CBF1	*CBF1(C-repeat binding factors)* are involved in cold response system	Loss of function	Decrease in chilling stress tolerance	Li et al. 2018a
	SlMAPK3	*MAPK s* (Mitogen-activated protein kinases) are the signaling molecules involved in drought stress	Loss of function	Decrease in drought stress tolerance	Wang et al. 2017
	Carotenoid cleavage dioxygenase8(CCD 8) More Auxiliary Growth1 (MAX1)	*CCD8 and MAX1* genes help synthesize strigolactones, which are branching inhibitory hormones and are required for germination of root parasitic weed	Loss of function	Resistance against *phelipanche aegytiaca*	Bari et al. 2021a
	MPK20	*SlMPK20* regulates the post-meiotic development of pollen by modulating sugar as well as auxin signaling and metabolism	Loss of function	Repression of genes controlling sugar and auxin metabolism	Chen et al. 2018
	Enzymes pectate lyase *(PL)*, polygalacturonase 2a (*PG2A*), and β −galactanase *(TBG4)*	*PL*, *PG2a*, and *TBG4* work on separate cell wall domains and are involved in shelf life.	Generation of a range of CRISPR alleles	Pectin degradation control	Wang et al. 2019
	ARF7	*ARF7* is the key repressor of the initiation of fruit in tomato	Loss of function	Parthenocarpic	Hu et al. 2018
	SlSTK	Serine/Threonine kinase domain regulates the signaling of glucose, which is essential for the plant for stress response, growth, and development	Loss of function	Attenuated sensitivity of glucose	Lu et al. 2023

Phospholipase C2 gene *SlPLC2* regulates the effect of pathogens in plants, resulting in resistance or susceptibility of plants towards different diseases. There are six members of PLC present in tomato *SlPLC1-SlPLC6*. CRISPR/Cas9 knockout of the *SlPLC2* gene decreased the production of ROS upon *B.cinerea* infection, which increased resistance towards the pathogen by reducing the pathogen proliferation as proliferation requires the production of ROS for cell death. Moreover, the *SlPLC2* mutant produced small necrotic areas (Perk et al. 2023). Late blight is one of the fungal diseases caused by *Phytophthora infestans*. Knockout of *SlMYBS2* reduced the resistance towards *P. infestans* by increasing the necrotic cells, disease index, and lesion size and decreasing the expression of the pathogenesis-related (PR) gene in the mutated plant, which concluded that *SlMYBS2* is a positive regulator for the *P. infestans* in tomato (Liu et al. 2021). Besides this, MicroRNA has a great role in susceptibility by targeting the resistant gene. CRISPR/Cas9 multiplex knockout of *miR482b* and *miR482C* shows more resistance towards *P. infestans* than the single knockout of *miR482b* (Hong et al. 2021). In potato, mutation of the *PMR4* gene reduced susceptibility to powdery mildew. An orthologue of the *PMR4* gene was found in tomatoes, and four different guide RNAs were designed to target different regions of the *SlPMR4* gene in two different varieties of tomatoes. Four sgRNAs successfully knocked out the *PMR* locus (Figure 3) and induced resistance towards *P.infestans* (Li et al. 2022a). Knock out of *NUCLEOREDOXIN* gene in tomato *SlNRX1* and *SlNRX2* exhibited resistance towards the pathogen *Pseudomonas syringae pv. maculicola (Psm) ES42326*, which causes bacterial leaf as well as towards the fungal pathogen *Alternaria brassicicola* in mutant *slnrx*. However, a mutation in *slnrx2* showed no resistance (Cha et al. 2023). Mutant *slnrx1* increased endogenous salicylic acid and decreased the jasmonic acid level after infection with *Psm* compared with *slnrx2* and wild-type plants. In the mutant *slnrx1*, the SA biosynthesis gene *SlICS1(ISOCHORISMATE SYNTHASE1)* and *SlEDS5 ENHANCED DISEASE SUSCEPTIBILITY 5* were upregulated compared to wild type plant. However, the *PR1* gene, a systemic acquired resistance gene, increased in *slnrx1* compared to the wild type (Cha et al. 2023). CRISPR activation of *SlPR-1 PATHOGENESIS-RELATED GENE 1* showed enhanced disease resistance against *Clavibacter michiganesis subsp.* without changing their agronomic character (García-Murillo et al. 2023). PTI (Pattern effector-triggered immunity) and ETI (effector-triggered immunity) are the two tired immune response systems in plants towards pests and pathogens. NLR receptor link with PTI and ETI, which is required for plant immune response. Mutation in *h-NLR SlNRC4a* and *SlNCR4b* resulted in a gain of constitutive

function defense activation and broad-spectrum disease resistance. The double mutant of *SlNRC4a* and *SlNRC4b* increased resistant against the fungal pathogen compared to a single mutation (Leibman-Markus et al. 2023). 3-Dehydroquinate dehydratase/shikimate dehydrogenase*(DQD/SDH)* is involves in the synthesis of shikimate. Knock out of *SlDQD/SDH2*, which is a ripening associated factor, lowers the content of flavonoids and shikimate by down-regulating flavonoid biosynthesis genes and shows resistance towards *Botrytis cinerea* (Wang et al. 2023).

3.6.3 Harnessing CRISPR/Cas9 to engineer virus resistance in tomato

Viruses that affect plants are usually obligate parasites that completely depend upon the host for survival. Moreover, the susceptible factors, also called recessive genes, present in the plant are the main factors that regulate viral disease in plants. Tomato yellow leaf curl virus (TYLCV) is from the Geminiviridae family. It causes one of the devasting viral diseases for tomatoes. *Ty-5* (*SlPelo*) is one of the susceptibility factors (S gene) for TYLCY. Knockdown of SlPelo through CRISPR/Cas genome editing confers host-mediated immunity against the pathogen (Praminik et al. 2021). Interestingly, a tomato plant with a stably designed CRISPR/Cas9 platform targeting viral genes encoding coat protein (*CP*) or replicase (*Rep*) demonstrated improved resistance to TYLCV infection. Translation initiation factor (*eIF4E*), a cap-binding protein, is one of the prominent susceptible factors to potyvirus. The recent CRISPR/Cas9-based knockdown of the eIF4E1 gene in tomatoes showed enhanced resistance to multiple viruses, including the cucumber mosaic virus, the pepper mottle virus, and the potato virus Y N strain (Yoon et al. 2020; Atarashi et al. 2020). The Pepino mosaic virus (PepMV) was first identified in 1999 as a tomato pathogen in the Netherlands. Since then, it has spread worldwide and created a pandemic in tomato crops, causing great economic loss. *SlGSTU38* is the susceptible gene for *PepMV* mutation, and this gene triggers the accumulation of oxygen species in leaves and deregulates stress-responsive genes (Mendez - Lopez et al. 2023).

3.6.4 Weed control in tomato

The obligate and facultative are the two characterized parasitic plants that adopt different forms for invading host plants by attaching them through their roots or shoots. Most economically significant crops are infected by root parasite weeds, which reduces yield and yield quality. Important agronomic crops are severely harmed by the parasitic weeds *Orobanche* and *Phelipanche* spp., which are entirely dependent on the host for their nutritional value. Strigolactones are plant hormones produced from plant carotenoids by cleavage of the CCD7 and CCD8 enzymes. These are necessary for germinating parasitic root weeds and function as a branching inhibitory hormone. CRISPR/Cas9 genome editing of two homologs of ATP binding cassette transporter (ABC) genes *Solyc08g067610* and *Solyc08g067620* in tomato decreased the growth of parasitic weeds *P. aegyptiaca*. It reduced the primary stem length, increased branching, and increased axillary bud growth. Moreover, the expression of two strigolactone biosynthetic genes, i.e., *CCD8* and *MAX1*, was significantly decreased in the ABC mutant lines and resulted in an alteration in root extract orobanchol (Bari et al. 2019). The mutated ccd8 in the second exon shows some morphological changes, like adventitious root formation and increased shoot branching. Additionally, *CCD8* mutants with SL-deficient show reduced parasite infection. It was observed that in the *CCD8* mutation, orobanchol (SL) content was reduced, and carotenoid and expression of genes that participate in carotenoid biosynthesis increased (Bari et al. 2019).

Further, the CRISPR/Cas 9 strategy has been successfully used to mutate the SL biosynthetic gene More Auxiliary Growth1 *(MAX1)* (Bari et al. 2021b). Due to lowered orobanchol levels, the *Slmax1* edited lines showed resistance to *P. aegyptiaca*. The study offers a fresh insight into creating an effective management strategy that might be utilized to prevent the growth of root parasite weeds, significantly impacting the agricultural economy (Bari et al. 2021a).

3.7 Engineering abiotic stress tolerance in tomato

Abiotic stresses include high or low temperature, excessive or inadequate water conditions, high salt concentration, accumulation of heavy metals, etc., affecting the plant's growth and development (Liu et al. 2022). Modifying the genes involved in the regulatory pathway of certain stress hormones, reactive oxygen species, etc., can create artificially resistant plants. CRISPR/Cas technologies help understand the crucial role of complex mechanisms of various abiotic stresses in plants and develop new climate-resilient crop varieties (Illouz-Eliaz et al. 2020).

3.7.1 Heat stress

Primarily due to global warming, as the temperature increases, it significantly affects the growth and development of plants. An increase in the production of ROS, cellular damage, and unwanted biological compound formation leads to a disturbance in plant cellular metabolism under heat stress (Chaudhury and Sidhu 2022). CRISPR/Cas9 strategy has been effectively utilized to tackle the heat stress response in tomatoes. Knocked out of *the SlBZR1 gene of the tomato showed susceptibility* to heat stress by reducing the quantum efficiency of photosystem II (Yin et al. 2018). Similarly, mitogen-activated protein kinases (MAPKs) are involved in multiple signaling pathways that lead to abiotic stress tolerance (Yu et al. 2019). CRISPR/Cas9-mediated mutation of *SlMAPK3* reported less cell membrane damage and ROS production, less

wilting, and more expression of heat shock proteins (HSPs) and heat shock factors (HSFs) (Yu et al. 2019).

3.7.2 Osmotic stress

Most abiotic stress like drought, salinity, and cold stress causes the plant's osmotic stress, where the water and electrolyte imbalance occurs. Osmotic stress damages the cellular and DNA labels, which has a major effect on the physiology of plant cells (Chen et al. 2021a). The tomato plant requires a lot of water, and any imbalance limits growth, germination, and elongation (Liu et al. 2018). As per the previous report, auxin response factors (ARFs) have an influential physiological role in plants (Roosjen et al. 2018). A gene of the ARF family *SlARF4*, expressed in guard cells and tomato's vascular bundle, was studied under water deficit conditions. In a study, it was reported that abscisic acid treatment and deficiency in water reduced the expression of the *SlARF4* gene (Bouzroud et al. 2018). CRISPR/Cas9 mediated loss of function mutation of the *SlARF4* gene resulted in enhanced tolerance to water stress and dehydration in the mutated lines (Chen et al. 2021b). This indicates that the *SlARF4* gene might be associated with the ABA signaling pathway by modulating the expression of *SlABI5/ABF* and *SCL3* genes, helping the tomato plant resist water deficit conditions (Chen et al. 2021a).

Similarly, in tomatoes, it was found that the PROCERA gene encodes the DELLA protein (a negative growth regulator) associated with the tomato resistance towards osmosis stress. Loss of function mutation of DELLA protein using CRISPR/Cas9 and a sgRNA provides several dominant dwarf mutations and loss-of-function mutations (Tomlinson et al. 2019). In 2019, the dominant dwarf PROCERA allele was first reported, showing partial responsiveness towards exogenously applied gibberellins. The intermediate phenotype was observed in heterozygotes at the seedling stage, but later, during their adult stage, the heterozygotes were dwarfed as homozygotes (Tomlinson et al. 2019). Based on these studies, it can be assumed that specific genetic networks regulate abiotic factors that monitor the osmotic stress in plants well, and with the advent of gene editing, resistant tomato varieties can be generated.

3.7.3 Drought stress

Multiple genes control drought stress in tomato CRISPR/Cas9 system was used to edit *SlNPR1* (Stable tomato *NPR1*) gene from tomatoes to study its role in regulating tomato drought tolerance response (Li et al. 2019). Reduced drought tolerance was evident in the *slnpr1* mutants, and it was further supported by the expression of genes associated with drought, such as *SlGST*, *SlDREB, and SlDHN*, being downregulated. *SlNPR1* mutants, by reducing the expression of multiple drought tolerance genes, also concurrently resulted in increased levels of malondialdehyde (MDA) and hydrogen peroxide, larger stomatal aperture, higher electrolytic leakage, and lower activity of antioxidant enzymes in the mutant lines (Table 1, Figure 3) (Li et al. 2019). In yet another study, CRISPR/Cas9 mutated *SlLBD40* knockouts were found to be less responsive to drought than WT tomato plants by increasing the water-holding ability (Liu et al. 2020b).

3.7.4 Chilling stress

The tomato plant is sensitive to chilling. In many species, it was found that highly conserved CBFs (C-repeat binding factors) are responsible for cold-response systems. The CRISPR-Cas9 generated *slcbf1* mutant demonstrated a more chilling-injury response than the wild type with high malondialdehyde and hydrogen peroxide contents, increased antioxidant enzymes, and higher electrolyte leakage (Li et al. 2018a). Low temperatures induce gene expression and protein content of SlNPR1. Mutation of *SlNPR1* resulted in chilling stress by increasing oxidative damage and synthesis of ferulic acid (Shu et al. 2023).

4 Challenges and Future prospects of tomato genome editing

Since the last decade, CRISPR/Cas9 genome editing technology has emerged as an evolutionary tool in agricultural biotechnology for crop improvement. However, the technology faces some major challenges that need to be addressed for further improvement and adoption in crop improvement.

4.1 Overcoming PAM Constraint

Mutation frequency is enhanced by minimalizing the distance between the cut or PAM sites and the edit sites. To address this problem, the idea of engineered Cas9 protein free from PAM site restriction has been developed. Recently developed Cas9 variants, SpG can efficiently cut at NGN sites where SpRY can act in almost every location near the target sites. SpRy is designed from wild variety Cas9 protein by changing 11 amino acids in its peptide chain. SpRy is potentially more efficient on NRN (R=A, G) PAM sites than NYN (Y=C, T) PAM sites in human cells. Genome editing in rice protoplast by SpRY revealed a similar fact with human cells that SpRy has the potential to edit all NNN PAM sites as well as a relatively high potential to edit at NRN PAM sites instead of NYN PAM sites (Ren et al. 2021).

4.2 Delivery Methods

Genome editing in plants has a few constraints, like unwanted insertion or deletion, off-target activity, sgRNA mismatch, and overexpression of Cas9 (Shen et al. 2019). Delivering editing machinery, i.e., gRNA or CRISPR/Cas9 components, into the host plant is still challenging. The common approaches are floral-dip-mediated transfer, Particle bombardment, Protoplast transformation, and *Agrobacterium* transformation. All these

methods have particular benefits as well as disadvantages. Dipping flower buds execute the floral dip method in a buffer containing *Agrobacterium*, then collecting seeds and selecting a positive transformant by growing them in a selection media. Similarly, *Agrobacterium*-mediated plant transformation is also executed by co-cultivating explants followed by positive transformant selection in a selection media. However, both methods require a thorough screening for T-DNA and Cas9-free mutant homozygous lines. Although particle bombardment and PEG-protoplast-mediated approaches are efficient, they also generate transgenic plants that must pass through extensive screening.

Viral vectors are most widely used in plant cells for gene silencing and the expression of inserted foreign proteins. Transgenic plants have already been edited with the expression of Cas9 nucleoprotein by using a viral vector system (Hu et al. 2019). However, transgene-free genome editing proves to be complicated due to certain virus restrictions. DNA and +ve strand RNA viruses were used in the plant genome for editing but are limited due to their cargo capacities (Liu et al. 2020a). The use of negative-strand RNA virus avoided the difficulties faced due to the use of positive-strand RNA virus as vector due to the large cargo capacity in negative-strand RNA virus vector. After continued effort, viral vectors are now intriguing tools for the transgenesis-free genome editing approach, which uses host machinery for their multiplication, enabling a broad level of expression (Cody and Scholthof 2019). Prior endeavors to express sgRNA using plant RNA virus vectors, such as tobacco mosaic virus, tobacco rattle virus, barley stripe mosaic virus, beet necrotic yellow vein virus, pea early browning virus, and foxtail mosaic virus, were successful in introducing mutations into host genomes (Ellison et al. 2020). However, due to the massive size of Cas9 and the negative correlation between the stability of plant viral vectors and the length of foreign gene insert, expression of virus vector-mediated Cas9 is difficult. Although the delivery of SYNV vector is possible, plant regeneration has certain limitations as plant rhabdoviruses are difficult to invade into plant germline or meristem cells (Liu et al. 2020b).

4.3 Transgene free genome editing

In conventional genome editing methods, transfer and a combination of DNA cassettes are needed to modify the host genome, which causes indels and changes in the DNA sequence and generates detrimental effects (Kim et al. 2014). However, transgene-free gene editing is fast and expanding due to its advantages. This technique targets genome alteration without any conflict with the gene and creates an opportunity for the delivery of non-genetically modified organisms (Rather et al. 2022). However, it has the same issue of transformation as in the conventional gene editing approach. The transformation approach mainly includes the invitro RNP complex comprising Cas9 and gRNA, which different approaches like microinjection, electroporation, particle bombardment, protoplast-mediated transformation, etc, can deliver (Zhang et al. 2021a). The other approach for transformation includes virus-mediated delivery and type IV secretory system of *A. tumefaciens* for Cas/gRNA delivery into plant cells (Tsanova et al. 2021). Ribonucleoprotein(RNPs) mediated genome editing is one of the methods for generating transgene-free genome editing plants in which the RNPs are composed of Cas9 nuclease protein and sgRNA, which will be delivered into the plant cell by using protoplast mediated transformation by using PEG, and calcium in rice and *Arabidopsis* (Toda et al. 2019). Particle bombardment was transformed to transfer the RNP into embryonic maize and wheat (Woo et al. 2015). However, the major disadvantage is full-length plant regeneration, and the selection process is time-consuming and expensive. Recently, DNA-free gene editing through protoplast-mediated transformation was performed in potatoes with 95% editing efficiency (Rather et al. 2022).

4.4 Grafting-mediated transformation of Cas9 and sgRNA cassette

Protoplast-mediated transformation is unsuitable in some plant species, or it takes a long time for a generation to overcome this problem. Designed Cas9 and gRNA transcripts are transferred from transgenic roots to shoots of wild-type plants (Scions) (Yang et al. 2023). Using such grafting, wild-type scions can be transferred into genome-edited mutants by transferring the gRNA and Cas9 transcript from the transgenic root. A tRNA-like sequence or TLS motifs were added along with Cas9 and gRNA transcript for movement from root to shoot.

4.5 Regulatory aspects of genome-edited tomato

Few nations, such as South and North America, have removed regulatory barriers to encourage the commercialization of GE crops. Furthermore, Australia and Japan have reviewed and updated their normative approval processes for genetically engineered organisms and goods, including site-directed nuclease 1 (SDN-1) type alteration (Kaul et al. 2020). Genome-edited crops developed using SDNs are categorized as SDN 1, 2, and 3. Most countries follow the SDN terminology to categorize the SDN applications legally. Oligonucleotide-directed mutagenesis (ODM) is also an alternative approach for targeted mutagenesis, and the outcome is mainly considered an SDN-2 event. In the last few years, several countries have adopted legislation and introduced guidelines for clarifying genome-edited products' legal status (Menz et al. 2020). CRISPR/Cas9 is subject to strict regulation in Europe, and the European Court of Justice included genome-edited crops in the rules governing genetically modified crops. Meanwhile, Australia allows genome editing without the involvement of foreign particles (Zhang et al. 2021b). Asia, China,

and Japan have relaxed restrictions on genome-edited crops, and there have been reports of such crops being grown in the field. The first gene-edited food that entered the market was GABA-enriched tomato. GABA is a health-promoting compound like vitamin C and a neurotransmitter that helps block impulses between nerve cells in the brain (Alamgir 2018). Tokyo-based Sanatech Seed has sold CRISPR/Cas9 genome-edited Sicilian Rouge tomatoes since September 2021. This genome-edited food contains a high amount of γ-aminobutyric acid (GABA), which helps to lower blood pressure and promote relaxation (Waltz 2022). A commercialized startup, Sanatech, from the University of Tsukuba, started sending free genome-edited tomato seedlings at the request of 4200 home gardeners in September 2021. More than 400 GABA-enriched food and beverage items, including chocolates, are already available in the Japanese market (Milon et al. 2024).

Conclusion

CRISPR/Cas9 is widely used as a promising tool for plant breeding. Its accuracy, simplicity, and precision make it a versatile system for modification of a wide range of crop plants. Recently developed prime editing system has advantages over base replacement and holds great promise in the precise modification of crops. Research endeavours must be adapted to successfully implement the CRISPR/Cas9 genome editing system in crop improvement, and the grand challenges must be addressed. Overcoming challenges like off-target activity, PAM constraint, genotype dependency transformation, and delivery methods can greatly increase crops' editing efficiency. Furthermore, the regulatory issues and ethical concerns associated with the technology must be debated globally, and scientific frameworks must be developed to widen the acceptance of edited crops.

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