

GENE EDITING IN THE DEVELOPMENT OF SWEET PEPPER HYBRIDS FOR OPEN-FIELD CULTIVATION: THE SIGNIFICANCE AND PROSPECTS OF APPLYING CRISPR/CAS9 AND ITS NEW-GENERATION TOOLS

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Abstract

Sweet pepper (*Capsicum annuum* L.) is a heat-loving, high-value vegetable crop whose yield and product quality are considerably limited by viral and fungal diseases, pests and abiotic stresses. Combining resistance and quality traits simultaneously in open-field hybrids by conventional breeding methods requires a long time and considerable labour. CRISPR/Cas9 and its new generations — Cas12a, base editing, prime editing and multiplex editing — allow precise, targeted modifications to be introduced into genes of interest and thus accelerate the breeding process. This review analyzes the main directions of gene editing in pepper — resistance to powdery mildew (CaMLO2), anthracnose (CaERF28, NAC72) and potyviruses (eIF4E/pvr2) — as well as the results achieved in improving fruit quality and agronomic traits. At present, the recalcitrance of pepper to in vitro regeneration remains the main technical obstacle; virus-induced gene editing (VIGE), DNA-free RNP methods and developmental regulators (GRF-GIF, BABY BOOM, WUSCHEL) are promising approaches to overcoming it. The article also discusses the regulatory status of transgene-free edited cultivars and the prospects for integrating gene editing into the breeding of open-field sweet pepper hybrids.

Keywords: CRISPR/Cas9, sweet pepper, *Capsicum annuum*, gene editing, base editing, prime editing, eIF4E, CaMLO2, virus resistance, hybrid breeding, transgene-free cultivars.

Introduction

Sweet pepper (*Capsicum annuum* L.) is an economically important vegetable crop of the Solanaceae family, widely grown throughout the world. As a rich source of vitamin C, carotenoids, flavonoids and antioxidants, it occupies an important place in the human diet.

In Central Asia, including Uzbekistan, sweet pepper is one of the main vegetable crops grown in open fields. A hot and dry climate, irrigation-dependent agrotechnology and strong pressure from viral and fungal diseases are the principal factors limiting the yield of this crop. As a result of climate change, the spread of pests and pathogenic microorganisms is intensifying, and crop losses may reach 20–40%.

Conventional breeding methods — introgression, hybridization and selection — are effective in combining valuable agronomic traits, but they require many years of work, large areas and considerable time. In addition, the limited natural gene pool of resistance genes and the problem of linkage drag complicate the development of elite sweet pepper hybrids. For this reason, the need for precise, rapid and targeted breeding technologies is growing.

The CRISPR/Cas9 system, described in 2012 as programmable “molecular scissors”, initiated revolutionary changes in plant genetics and breeding. In subsequent years, the improved generations of this system — Cas12a (Cpf1), base editing and prime editing — have made it possible to introduce precise point mutations without double-strand breaks (DSBs) and without the need for a foreign DNA donor. This review article analyzes the current state of gene editing technologies in sweet pepper breeding, their specific technical limitations and their prospects for the development of open-field hybrids.

Fundamentals of CRISPR/Cas9 technology and its new generations

Mechanism of action. The CRISPR/Cas9 system consists of two main components: a single guide RNA (sgRNA) and the Cas9 endonuclease. The sgRNA recognizes the target sequence in the genome through complementary base pairing and directs the Cas9 protein to the precise site. Targeting requires a PAM (protospacer adjacent motif; 5'-NGG-3' for *Streptococcus pyogenes* Cas9) sequence adjacent to the target site. Cas9 cleaves both DNA strands, creating a double-strand break (DSB).

The resulting break is repaired through the cell's natural repair pathways: non-homologous end joining (NHEJ) produces small insertions/deletions (indels) and gene knockouts, whereas homology-directed repair (HDR) enables precise substitutions in the presence of a donor template. Because the efficiency of HDR is low in plants, most practical work employs the NHEJ pathway, which is based on loss of gene function.

Cas12a and the expanded nuclease arsenal. Alongside Cas9, the Cas12a (Cpf1) nuclease is also widely used. It recognizes a T-rich (5'-TTTV) PAM sequence, produces staggered (sticky-end) breaks and simplifies multiplex editing of several genes with a single crRNA. In studies carried out on pepper protoplasts, Cas9 and Cas12a ribonucleoprotein (RNP) complexes successfully edited the CaMLO2 gene.



Base editing. Base editing is a technology for precisely substituting a single nucleotide without creating a DSB. A Cas9 with reduced catalytic activity (nCas9 or dCas9) is fused to a deaminase enzyme: a cytosine base editor (CBE) performs the C·G → T·A conversion, whereas an adenine base editor (ABE) performs the A·T → G·C conversion. Because this approach requires no foreign DNA donor and reduces the risk of unintended mutations caused by double-strand breaks, it is particularly valuable for fine-tuning quality traits. Precise editing of the NAC72 locus by CBE has been tested in hot pepper as a means of enhancing anthracnose resistance.

Prime editing. Prime editing, based on the “search-and-replace” principle, makes it possible to carry out all twelve types of base conversion as well as precise insertions and deletions without double-strand breaks and without donor DNA. The system consists of an nCas9–reverse transcriptase fusion and a prime editing guide RNA (pegRNA) that contains the editing template. In plants, the efficiency of prime editing remains low and unstable — the main obstacle limiting its wide application — but optimization strategies are steadily improving it.

Multiplex and epigenome editing. Using several sgRNAs in a single construct to edit multiple genes or a group of genes simultaneously (multiplex editing) is effective when working with polygenic traits and gene families. Epigenome editing, in turn, makes it possible to regulate gene expression (through methylation or chromatin remodelling) without altering the DNA sequence. These approaches are promising directions for controlling complex agronomic traits.

Specific technical obstacles to gene editing in pepper

Although gene editing technologies are advancing rapidly in other Solanaceae crops such as tomato (*Solanum lycopersicum*), their application in pepper remains considerably limited. The main reasons are the recalcitrance of pepper to regeneration in vitro and the low efficiency of *Agrobacterium tumefaciens*-mediated transformation. High-value crops such as cotton, sunflower and pepper still remain among the species that are difficult to regenerate.

In many cases the limiting step is not the transformation of the cells itself, but the recovery of first-generation (T0) regenerant plants. Several innovative approaches are being developed to overcome this obstacle:

Virus-induced gene editing (VIGE). Virus-induced gene editing (VIGE) is considered promising as an in planta editing method that does not require tissue culture. An sgRNA was delivered through a vector based on tobacco rattle virus (TRV2) into transgenic pepper seedlings constitutively expressing Cas9, and editing of the Phytoene desaturase (PDS) gene produced a photobleaching phenotype. To enhance the mobility of the sgRNA, a self-cleaving hammerhead ribozyme and a fragment of the mobile RNA of FLOWERING LOCUS T were incorporated into the vector. Systemic and heritable editing was achieved with this method.

DNA-free RNP and protoplast systems. Mixing purified Cas9 or Cas12a enzyme with an sgRNA and delivering it into protoplasts as an RNP complex by means of PEG enables DNA-free editing. This approach leaves no foreign gene residues and is therefore advantageous from

a regulatory point of view. A stable screening system was developed for the efficient editing of the CaMLO2 gene in the fully sequenced *C. annuum* cultivars “CM334” (hot) and “Dempsey” (sweet). Protoplast-based sgRNA selection allows efficient guide RNAs to be tested rapidly before regeneration.

Developmental regulators. Expressing morphogenic regulators is an effective strategy for overcoming the regeneration barrier. A chimeric protein (GRF-GIF) composed of GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) increased regeneration efficiency in wheat, rice, citrus and tomato, almost doubling the number of T0 plants without any appreciable adverse effect on growth. Co-expression of the BABY BOOM (BBM) and WUSCHEL (WUS) genes also stimulates regeneration. Importantly, expression of the BnBBM gene has been shown to induce abundant cell regeneration specifically in sweet pepper (*Capsicum annuum*) — a promising direction for this crop.

Main directions in the application of CRISPR to sweet pepper breeding

Resistance to fungal diseases. Powdery mildew is a serious disease of pepper both in greenhouses and in open fields. The Mildew Locus O (CaMLO2) gene plays an important role in the plant's defence response to fungal pathogens; knocking out this susceptibility gene enhances resistance. In a comparative study carried out on six commercial cultivars, the Cas9/CaMLO2sgRNA1-RNP system achieved an editing efficiency of 6.3–17.7% regardless of cultivar genotype.

Anthrachnose (*Colletotrichum* spp.) is the most damaging disease of pepper in tropical and subtropical regions. Knockout of the CaERF28 (ethylene response factor) gene by means of a single transcript unit (STU) CRISPR/Cas9 system enhanced anthracnose resistance in hot pepper. Precise editing of the NAC72 locus by cytosine base editing (CBE) has also been tested as a promising approach against anthracnose.

Virus resistance. Potyviruses (Potyviridae) account for approximately 30% of known plant viruses and pose a serious threat to pepper (for example, Chilli veinal mottle virus, Pepper mottle virus, Pepper veinal mottle virus). Many recessive virus resistance genes encode eukaryotic translation initiation factors (eIF4E, eIF4G). The eIF4E protein is exploited by the virus as a susceptibility factor for its multiplication; modifying it confers resistance.

In pepper, 25 natural allelic variants of the eIF4E1 gene have been identified, and they are linked through the pvr2 locus to resistance against various potyviruses. These naturally resistant alleles frequently carry point substitutions in regions I and II surrounding the cap-binding pocket of the eIF4E1 protein, particularly at amino acid positions 66–68 (P66T, V67E, A68E). Reproducing precisely this natural polymorphism by base editing is a promising route to generating potyvirus resistance in an accurate and transgene-free manner. The fact that CRISPR knockout of the eIF4E1 and eIF4E2 genes in tomato conferred resistance to potyviruses confirms that this strategy can also be applied to pepper.

Fruit quality and agronomic traits. Phytoene desaturase (PDS) gene is involved in carotenoid biosynthesis and is often used as a marker gene giving a visible phenotype (photobleaching) when optimizing editing protocols. In the wider Solanaceae, gene editing has been successfully applied to improve quality traits such as increasing lycopene content, parthenocarp (seedless fruit set), extending fruit shelf life (by knocking out pectin-degrading enzymes) and reducing enzymatic browning. These approaches can be directed towards the targeted improvement of traits in sweet pepper such as fruit colour, vitamin C and carotenoid composition, storability and marketable appearance.

Abiotic stress tolerance. Under Central Asian conditions, heat, drought and soil salinity are the main abiotic factors affecting open-field pepper. Targeted editing of stress-response genes, transcription factors and genes associated with hormonal signalling pathways is a promising direction for developing heat- and drought-tolerant cultivars. Results obtained in other vegetable crops (for example, salt tolerance in squash) provide a conceptual basis for pepper as well, although dedicated research in this direction in pepper is still at an early stage.

Next-generation editing and precision breeding

While classical CRISPR/Cas9 is adapted mainly to gene knockout, base editing and prime editing raise precision breeding to a new level. Reproducing natural resistance alleles exactly (for example, the substitutions in region I of eIF4E1) reduces the risk of unintended mutations caused by double-strand breaks and of complete loss of gene function. This is particularly important in cases where only the interaction with the virus must be disrupted while gene function is preserved.

Technically, base and prime editing require a larger number of independent transgenic events and more efficient analytical systems than classical CRISPR/Cas9. Therefore, in crops recalcitrant to regeneration such as pepper, combining them with developmental regulators such as GRF-GIF may be the key solution for applying these advanced editing methods in practice. Multiplex editing, in turn, makes it possible to knock out several susceptibility genes simultaneously and thus to create broad-spectrum and durable resistance.

Regulatory and biosafety aspects

The commercialization of gene-edited cultivars depends on their legal status. Transgene-free edited plants — that is, cultivars that contain no foreign DNA residues and carry only point mutations or small indels — are being assessed in many jurisdictions as close to the products of conventional mutagenesis. For example, under the European Union's proposal on New Genomic Techniques (NGT-1), an NGT product may be considered equivalent to conventional plants if it differs from the parent cultivar by no more than 20 modifications of certain defined types (≤ 20 nucleotide substitutions/insertions, deletions of any number, etc.).

DNA-free RNP methods and the generation of transgene-free edited plants through grafting facilitate compliance with these regulatory requirements. For Uzbekistan and the countries of Central Asia, establishing a clear regulatory and legal framework for gene-edited cultivars is a prerequisite for introducing these technologies into practical breeding.

Prospects for open-field sweet pepper hybrids

Gene editing does not supersede hybrid (F1) breeding; it complements it. Resistance and quality traits can be introduced into elite parental lines by targeted editing, and these lines can then be used in conventional hybridization schemes. This approach circumvents the problem of linkage drag and makes it possible to improve a particular trait precisely while preserving the genetic background of a cultivar.

The promising directions for open-field sweet pepper hybrids are as follows: (1) broad-spectrum virus resistance based on eIF4E1/pvr2; (2) resistance to powdery mildew and anthracnose through CaMLO2 and CaERF28; (3) improvement of fruit quality and of carotenoid and vitamin C content; (4) heat and drought tolerance. Combining these traits in a single cultivar through base/prime editing and multiplex approaches is becoming a realistic possibility.

At the same time, the key condition for realizing these prospects in pepper is to increase the efficiency of regeneration and transformation. Optimizing developmental regulators such as GRF-GIF and BBM/WUS, as well as VIGE and DNA-free methods, for local elite cultivars is the principal scientific and practical task facing breeding programmes in Central Asia.

Conclusion

CRISPR/Cas9 and its new generations — Cas12a, base editing, prime editing and multiplex editing — are powerful tools for improving resistance and quality traits in sweet pepper breeding precisely, rapidly and in a targeted manner. The initial results achieved in pepper for disease resistance (CaMLO2, CaERF28, NAC72) and virus resistance (eIF4E/pvr2) confirm the practical potential of the technology. At the same time, the recalcitrance of pepper to regeneration remains the main technical obstacle; VIGE, DNA-free RNP methods and developmental regulators (GRF-GIF, BBM, WUS) are promising solutions for overcoming it. The favourable regulatory status of transgene-free edited cultivars broadens the possibilities for integrating these technologies into open-field hybrid breeding. Under the conditions of Central Asia and Uzbekistan, the development of efficient editing and regeneration protocols for local elite cultivars should be a priority direction for future research.

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