

GENE EDITING IN THE DEVELOPMENT OF WHITE CABBAGE HYBRIDS FOR OPEN-FIELD CULTIVATION: THE SIGNIFICANCE AND PROSPECTS OF APPLYING CRISPR/Cas9 AND ITS NEW-GENERATION TOOLS

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Abstract

White cabbage (*Brassica oleracea* var. *capitata* L.) is a widely grown vegetable crop of high nutritional value, and the breeding of its open-field hybrids is associated with a number of biological and technical difficulties. Cabbage is a biennial plant with strong self-incompatibility that requires prolonged vernalization in order to flower; these characteristics complicate conventional mutagenesis and breeding. CRISPR/Cas9 and its new generations — Cas12a, base editing, prime editing and multiplex editing — allow targeted, precise modifications to be introduced and thus accelerate hybrid breeding. This review

analyzes the main directions of gene editing in cabbage: improvement of hybrid seed production (overcoming self-incompatibility through BoSRK and creating male sterility through BoMS1), disease resistance (black rot, clubroot, TuMV), and the control of product quality and glucosinolate composition (MYB28, CER1). The genotype-dependent response of cabbage to in vitro regeneration remains the main technical obstacle; protoplast-RNP systems, extended tissue culture and haploid inducer (CENH3) approaches are promising means of 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 white cabbage hybrids.

Keywords: CRISPR/Cas9, white cabbage, Brassica oleracea, gene editing, self-incompatibility, BoSRK, BoMS1, male sterility, hybrid seed production, black rot, clubroot, MYB28, glucosinolate, transgene-free cultivars.

Introduction

White cabbage (*Brassica oleracea* var. *capitata* L.) is one of the most widely cultivated vegetable crops in the world and belongs to the family Brassicaceae (crucifers). It is a rich source of vitamins (C, K, folic acid), minerals (calcium), flavonoids and health-promoting glucosinolates.

In Central Asia and Uzbekistan, white cabbage is one of the main vegetable crops grown in open fields. The principal factors limiting its yield and marketable quality are bacterial black rot, clubroot, fusarium wilt, viruses and pests, as well as abiotic stresses such as heat and drought. Although F1 hybrid cultivars are distinguished by their uniformity, yield and resistance, their development is a long and complex process.

The biology of cabbage creates particular difficulties from the standpoint of gene editing. First, cabbage is a biennial plant that requires prolonged vernalization at low temperature in order to flower. Second, it possesses strong self-incompatibility (sporophytic self-incompatibility). These characteristics considerably complicate the creation of a new gene pool through physical or chemical mutagens. Third, many genes in the *B. oleracea* genome are present as paralogous copies, which sometimes requires several copies to be knocked out simultaneously.

CRISPR/Cas9 and its improved generations are overcoming these limitations and making it possible to introduce precise, rapid and targeted modifications into cabbage breeding. This review article analyzes the current state of gene editing technologies in white cabbage breeding, their specific technical features and their prospects for the development of open-field hybrids.

Fundamentals of CRISPR/Cas9 technology and its new generations

The CRISPR/Cas9 system consists of a single guide RNA (sgRNA) and the Cas9 endonuclease. The sgRNA recognizes the target sequence in the genome and directs Cas9 to the precise site; targeting requires an adjacent PAM (5'-NGG-3') sequence. Cas9 creates a double-strand break (DSB), which results either in small insertions/deletions (indels) and gene knockout through non-homologous end joining (NHEJ), or in precise substitution through homology-directed repair (HDR) (Figure 1).

Figure 1. The CRISPR/Cas9 system and its new generations

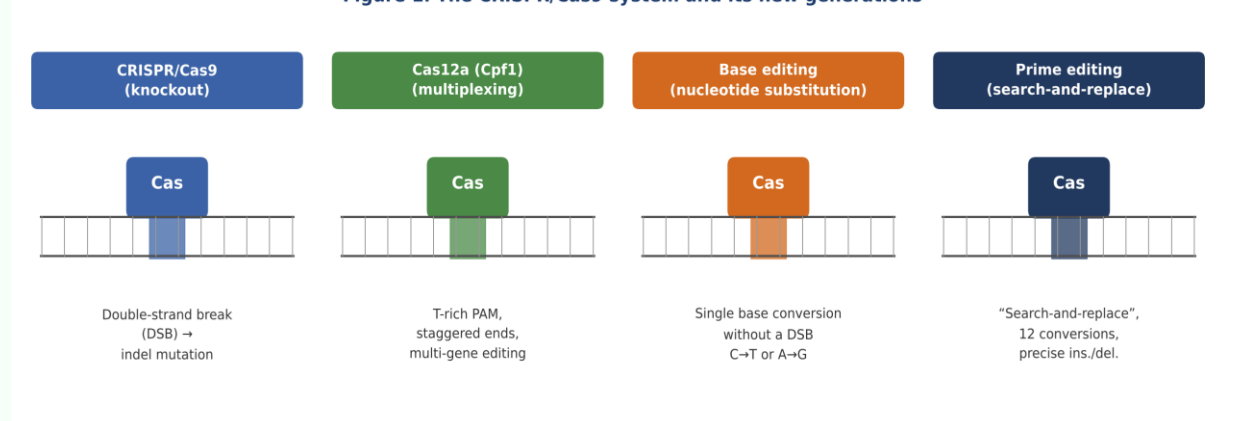


Figure 1. The CRISPR/Cas9 system and its new generations: Cas9 (knockout), Cas12a (multiplexing), base editing and prime editing.

Alongside Cas9, the Cas12a (Cpf1) nuclease recognizes a T-rich (5'-TTTV) PAM and simplifies multiplex editing of several genes with a single crRNA. Base editing substitutes a single nucleotide precisely without creating a DSB: a cytosine base editor (CBE) performs the C·G → T·A conversion and an adenine base editor (ABE) the A·T → G·C conversion. Prime editing, based on the “search-and-replace” principle, carries out all twelve types of base conversion as well as precise insertions and deletions without donor DNA. A comparative description of these generations is given in Table 1.

Table 1. Comparative characteristics of the generations of gene editing technologies

Technology	Mechanism	Main application	Limitations
CRISPR/Cas9	DSB → NHEJ/HDR	Gene knockout, indels	Unintended mutations, off-target effects
Cas12a (Cpf1)	T-rich PAM, staggered ends	Multiplex editing	Low efficiency at some loci
Base editing	nCas9 + deaminase	Precise point substitution (C→T, A→G)	Limited to certain bases
Prime editing	nCas9 + reverse transcriptase, pegRNA	All 12 base conversions, insertions/deletions	Low efficiency in plants

Specific technical features of gene editing in cabbage

The first successful results of gene editing in the genus *Brassica* were limited. Later, once the cabbage genome had been sequenced, a CRISPR/Cas9 method based on the endogenous tRNA-processing system made it possible to generate highly efficient and heritable mutations in cabbage. The transformation of *B. oleracea* is strongly genotype-dependent, and the crop remains among those that are recalcitrant to regeneration.

Protoplast and DNA-free RNP systems. Protoplasts with high yield and viability were isolated from two cabbage cultivars, and PEG-mediated delivery of CRISPR/Cas9 was optimized. Shoot regeneration required a high cytokinin concentration: a combination of 2.2 mg/L TDZ and 0.5 mg/L NAA provided up to 45% shoot regeneration. Delivering purified Cas9/Cas12a enzyme together with an sgRNA as an RNP complex enables DNA-free editing, leaves no foreign gene residues and is advantageous from a regulatory point of view.

Duration of tissue culture and haploid inducers. Editing efficiency depends on tissue culture conditions: maintaining *B. oleracea* calli on regeneration medium for seven weeks gave higher editing efficiency (without off-target effects) than four weeks. In genotypes recalcitrant to regeneration, editing of the *CENH3* gene is a promising approach for producing haploids, allowing transgene-free edited

plants to be generated through haploid inducer lines carrying the CRISPR reagents.

Main directions in the application of CRISPR to white cabbage breeding

The main directions of gene editing in cabbage and the target genes are summarized in Table 2. They can be divided into three groups: hybrid seed production, disease resistance, and quality and agronomic traits.

Table 2. Target genes for gene editing in white cabbage and related Brassica crops

Gene	Function	Editing outcome / direction
<i>BoSRK</i>	S-receptor kinase (stigma determinant)	Loss of self-incompatibility → self-compatible line
<i>BoMS1</i>	Male fertility (tapetum)	Completely male-sterile line → hybrid seed production
<i>CENH3</i>	Centromeric histone	Haploid inducer → transgene-free editing
<i>eIF(iso)4E</i>	Translation initiation factor	Resistance to turnip mosaic virus (TuMV)
<i>MYB28</i>	Aliphatic glucosinolate biosynthesis	Control of glucosinolate composition (quality)
<i>CER1</i>	Leaf cuticular wax biosynthesis	Reduced wax, leaf colour (morphology)
<i>BoPDS</i>	Phytoene desaturase (carotenoids)	Marker gene (protocol optimization)

Improvement of hybrid seed production. Hybrid (F1) seed production in cabbage is the most promising and distinctive field of application for gene editing. Conventionally, self-incompatible lines carrying different S-alleles are used to produce F1 hybrids, but the instability of self-incompatibility creates problems. The S-receptor kinase (SRK) gene is the principal stigma determinant of self-incompatibility in cabbage. Knockout of the *BoSRK3* gene completely abolished self-incompatibility and converted the line into a self-compatible one.

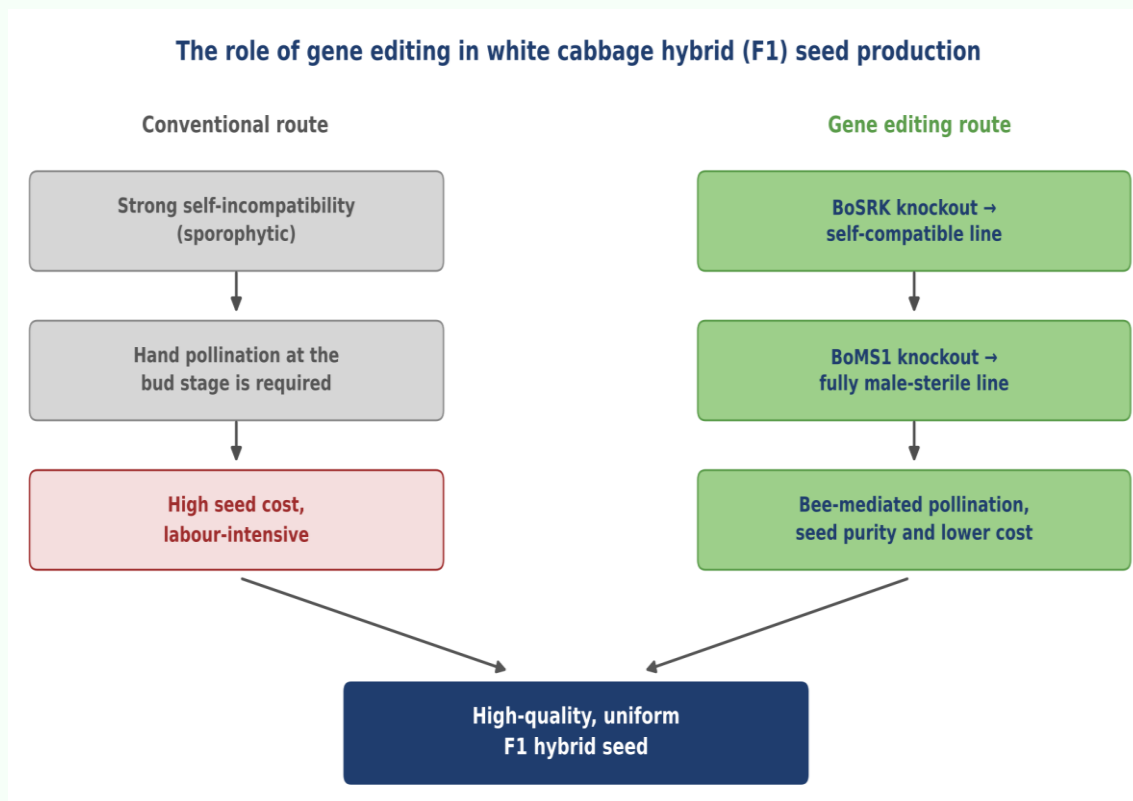


Figure 2. The role of gene editing in white cabbage hybrid seed production: reducing seed cost by editing the BoSRK and BoMS1 genes.

In addition, knockout of the *BoMS1* gene produced a completely male-sterile mutant; at the same time, owing to the *BoSRK3* mutation, this mutant showed high crossability with its non-mutant isoline at flowering — which made it possible to propagate the male-sterile line economically through bee-mediated pollination. Editing both genes simultaneously by multiplex editing considerably reduces the cost of hybrid seed production (Figure 2).

Disease resistance.

Black rot (bacterial black rot) — caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is the most damaging disease of cabbage and can reduce yield by up to 50%. It is a seed-borne disease that damages the vascular tissues. Resistance-associated loci (for example, *Bol031422*, the *4CL* gene family) and glucosinolate-regulated defence mechanisms have been identified; their targeted editing is a promising direction for developing resistant cultivars.

Clubroot (*Plasmodiophora brassicae*) is a serious disease that damages the roots of crucifers. A number of resistance genes (CRa, CRb, CRc, Crr1, Crr2 and others) have been introgressed from *B. rapa* into *B. oleracea*. Multiplex CRISPR editing makes it possible to strengthen resistance by pyramiding several R-genes in a single generation and thus to prevent its rapid breakdown.

Virus resistance. Turnip mosaic virus (TuMV) is the most widespread potyvirus damaging Brassica crops. In Chinese cabbage (*B. rapa*), editing of the *eIF(iso)4E* gene by CRISPR/Cas9 conferred resistance to TuMV; the absence of virus particle accumulation in the edited T₁ plants was confirmed by ELISA analysis, and a strong negative correlation was found between editing frequency and resistance ($r = -0.938$). This approach can also be applied to white cabbage.

Head/product quality and agronomic traits

Glucosinolates are secondary metabolites associated with the characteristic flavour of cabbage, its defence mechanisms and its health-promoting properties. The *MYB28* transcription factor is the principal regulator of aliphatic glucosinolate (A-GSL) biosynthesis, and its editing by CRISPR/Cas9 demonstrated control of glucosinolate composition under field conditions. Knockout of the *CER1* gene reduced the leaf cuticular wax layer and altered leaf colour. In addition, editing of glucosinolate transporter (*GTR*) genes has been applied to control quality traits in related Brassica crops. These approaches can be directed towards the targeted improvement of head density, storability, marketable appearance and nutritional composition in white cabbage.

Abiotic stress tolerance

Heat, drought and soil salinity are the main abiotic factors for open-field cabbage under Central Asian conditions. 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, although dedicated research in this field in cabbage 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 or quality alleles exactly reduces the risk of unintended mutations caused by double-strand breaks. Multiplex editing, in turn, makes it possible to edit several genes simultaneously — for example, BoSRK and BoMS1 for hybrid seed production, or a group of R-genes for disease resistance.

Gene editing does not supersede hybrid (F1) breeding; it accelerates and complements it. Using elite lines into which a trait has been introduced by targeted editing in conventional hybridization schemes circumvents the problem of linkage drag and considerably shortens the time required to develop a new cultivar (Figure 3).

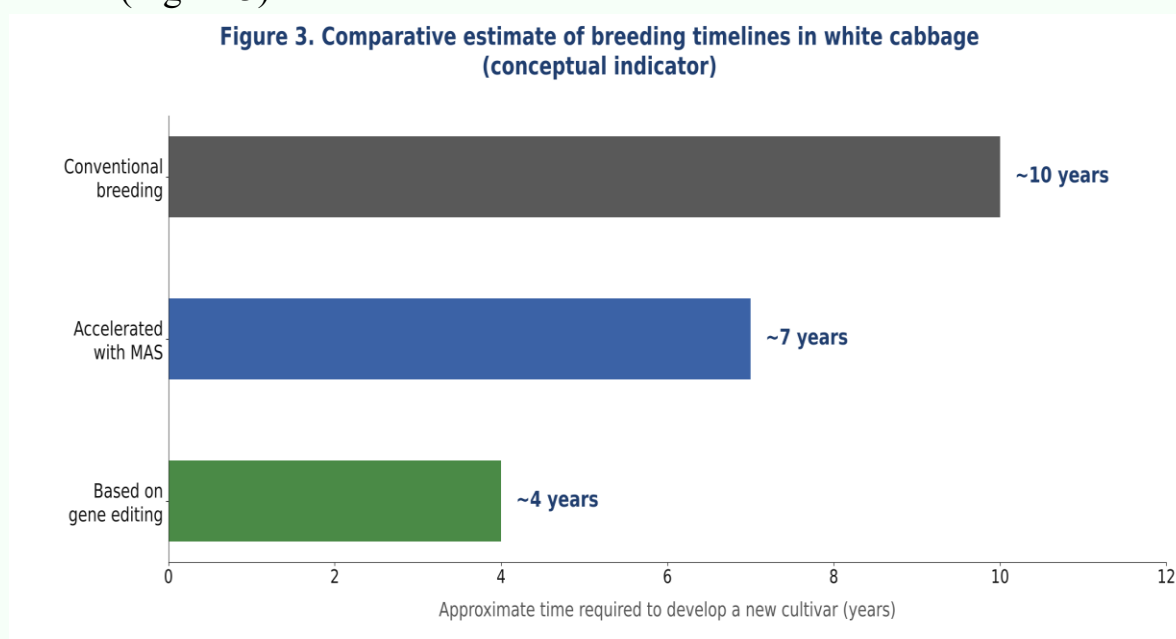


Figure 3. Comparative estimate of breeding timelines in white cabbage (conceptual indicator; the actual duration depends on the cultivar, the trait and the method).

Regulatory and biosafety aspects

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. Under the European Union's proposal on New Genomic Techniques (NGT), an NGT product that differs by modifications of certain defined types and in limited number may be considered equivalent to conventional plants. Generating transgene-free edited cabbage by the DNA-free RNP method and through haploid inducers facilitates compliance with these regulatory requirements. For

Uzbekistan and 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 white cabbage hybrids

The promising directions for open-field white cabbage hybrids are as follows: (1) simplifying and reducing the cost of hybrid seed production by editing *BoSRK* and *BoMS1*; (2) strengthening resistance to black rot, clubroot and TuMV through the pyramiding of R-genes; (3) improving glucosinolate composition, head density and storability through *MYB28* and other genes; (4) heat and drought tolerance. Combining these traits in a single cultivar through multiplex and base/prime editing is becoming a realistic possibility.

At the same time, the key condition for realizing these prospects in cabbage is to optimize the efficiency of regeneration and transformation for local elite cultivars. Adapting protoplast-RNP systems, extended tissue culture, haploid inducers and developmental regulators to the breeding programmes of Central Asia is a priority task for future research.

Conclusion

CRISPR/Cas9 and its new generations — Cas12a, base editing, prime editing and multiplex editing — are powerful tools for improving hybrid seed production, disease resistance and product quality in white cabbage breeding precisely, rapidly and in a targeted manner. The improvement of hybrid seed production through editing of the *BoSRK* and *BoMS1* genes is a particularly distinctive and economically important field of application for this technology. At the same time, the genotype-dependent recalcitrance of cabbage remains the main technical obstacle; protoplast-RNP, extended tissue culture and haploid inducer approaches are promising means of 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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