

GENE EDITING IN THE DEVELOPMENT OF CUCUMBER HYBRIDS FOR GREENHOUSE CULTIVATION: THE SIGNIFICANCE OF CRISPR/Cas9 AND ITS NEXT-GENERATION TOOLS

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Abstract

Greenhouse production of cucumber (*Cucumis sativus* L.) requires hybrids that combine stable femaleness, parthenocarpy, durable disease resistance and high fruit quality, whereas conventional breeding of such hybrids is slow. This review summarises the CRISPR/Cas9 toolbox and its next-generation derivatives (Cas12a, Cas13, base editors, prime editors and dCas-based regulators) and maps published studies onto the key greenhouse traits: gynoecey (CsWIP1, CsACS), parthenocarpy, resistance to powdery mildew (CsaMLO8, CsaMLO1/11), potyviruses (eIF4E) and downy mildew, and non-bitter fruit (Bi, Bl, Bt). The integration of editing into hybrid seed production and the main constraints (transformation efficiency, off-target risk, delivery and regulation) are discussed. Combined with speed breeding and genomics-assisted selection, these tools can substantially shorten the development of greenhouse cucumber hybrids.

Keywords: *Cucumis sativus*; CRISPR/Cas9; genome editing; base editing; prime editing; greenhouse cucumber; parthenocarpy; gynoecey; disease resistance; hybrid breeding.



Introduction

Cucumber (*Cucumis sativus* L., $2n = 2x = 14$) is a globally important cucurbit vegetable and a mainstay of greenhouse and plastic-tunnel production. The reference genome (Huang et al., 2009) made cucumber a genomic model among the Cucurbitaceae and laid the basis for molecular breeding, including genome editing. Protected cultivation sets specific breeding priorities. Because pollinators are usually absent, greenhouse types must be strongly gynoeceous and parthenocarpic. The warm, humid and dense crop favours powdery mildew, downy mildew and aphid-transmitted viruses, so durable resistance is decisive. The market also demands uniform, non-bitter, dark-green fruit with good shelf life. Combining all these traits in elite F1 parents by phenotypic selection and marker-assisted backcrossing is slow and is limited by the scarcity of favourable natural alleles.

CRISPR/Cas9 editing (Jinek et al., 2012; Cong et al., 2013) modifies specific loci without the linkage drag of introgression. Since the first transgene-free, CRISPR-derived virus resistance in cucumber (Chandrasekaran et al., 2016), many greenhouse traits have proved editable, and the toolbox has expanded to base editors, prime editors and transcriptional regulators (Chen et al., 2019; Zhang et al., 2019). This review assesses the significance of these tools for greenhouse cucumber hybrids.

The CRISPR/Cas Genome-Editing Toolbox and Its Next Generations

Cas9 and alternative effectors. A single guide RNA directs Cas9 to a ~20-nucleotide target next to an NGG protospacer-adjacent motif (PAM), where it makes a double-strand break (DSB). Repair by non-homologous end joining creates indels that usually knock out the gene; homology-directed repair with a donor allows precise insertion (Jinek et al., 2012; Cong et al., 2013). Cas12a recognises T-rich PAMs and processes its own crRNA array, which is convenient for stacking several traits in one event (Zetsche et al., 2015). Cas13 targets RNA and offers genome-neutral resistance to RNA viruses (Abudayyeh et al., 2017).

Base and prime editing. Base editors fuse a Cas nickase or dead Cas to a deaminase and change single bases without a DSB: cytosine base editors convert C·G to T·A (Komor et al., 2016), and adenine base editors convert A·T to G·C (Gaudelli et al., 2017). Prime editors couple a Cas9 nickase with a reverse transcriptase, and the prime-editing guide RNA (pegRNA) templates all twelve base substitutions as well as small insertions and deletions (Anzalone et al., 2019). For cucumber, these tools can convert susceptibility alleles into resistant ones and fine-tune quality genes without complete loss of function, although prime-editing efficiency in plants is still low.

CRISPRa, CRISPRi and transgene-free recovery. Catalytically dead Cas9 (dCas9) fused to activators, repressors or chromatin modifiers allows graded regulation of endogenous genes, for example dosage-sensitive sex genes or fruit-quality genes. For commercial breeding, edited plants free of foreign DNA can be recovered by segregating away the editing construct or by delivering transient ribonucleoprotein (RNP) complexes. Transgene-free edited cucumber was obtained by Chandrasekaran et al. (2016) and Hu et al. (2017). This status supports consumer acceptance and, in many countries, a more favourable regulatory classification.

Table 1. The CRISPR/Cas genome-editing toolbox and its relevance to cucumber breeding. DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair; PAM, protospacer-adjacent motif; CBE/ABE, cytosine/adenine base editor.

Effector / editor	Target & mechanism	Type of edit	Key advantage for cucumber breeding	Key reference(s)
Cas9	dsDNA; RNA-guided blunt DSB (NGG PAM), repaired by NHEJ or HDR	Knockout (indels); knock-in with donor	Simple retargeting and multiplexing of validated loci	Jinek et al., 2012; Cong et al., 2013
Cas12a (Cpf1)	dsDNA; staggered cut, T-rich PAM; processes its own crRNA array	Knockout; efficient multiplex editing	Single-vector stacking of several traits in one event	Zetsche et al., 2015
Cas13	ssRNA; RNA-guided cleavage	Post-transcriptional interference (no genome change)	Genome-neutral resistance to RNA viruses	Abudayyeh et al., 2017
Base editors (CBE, ABE)	n/dCas + deaminase; edits within a window, no DSB	C·G→T·A (CBE); A·T→G·C (ABE)	Convert susceptibility alleles to resistant ones; recreate favourable SNPs	Komor et al., 2016; Gaudelli et al., 2017
Prime editor	nCas9 + reverse transcriptase; pegRNA templates the edit	All 12 substitutions; small insertions/deletions	Precise allele engineering of quality and resistance traits	Anzalone et al., 2019
dCas9 (CRISPRa / CRISPRi)	Binds without cutting; activator, repressor or chromatin-modifier fusion	Graded up-/down-regulation; heritable epigenetic change	Tune dosage-sensitive sex and cucurbitacin genes	Chen et al., 2019; Zhang et al., 2019

The Breeding Demands of Protected Cucumber Production

In protected cultivation the value of a hybrid depends on a few high-stakes traits: at high plant density a single susceptibility can be catastrophic, and continuous harvests make uniform fruit set decisive. The greenhouse ideotype is a stably gynoeceous, parthenocarpic plant with durable, preferably recessive resistance and uniform, non-bitter fruit. These traits are difficult for conventional breeding: sex expression is unstable, S-gene resistance is recessive and laborious to fix, and non-bitterness depends on a biosynthetic gene cluster. Editing a defined locus achieves in one or two generations what backcrossing achieves in many, directly in the elite background (Chen et al., 2019).

Editable Trait Targets in Greenhouse Cucumber Hybrids

Sex expression and gynoecy. Sex determination in cucumber is controlled mainly by ethylene biosynthesis and signalling. The Female (F) locus, associated with CsACS1G, promotes femaleness in a dosage-dependent way, which makes gynoecy unstable in inbred lines. Natural loss-of-function alleles of CsWIP1, a suppressor of carpel development, are absent from cultivated germplasm, so editing is the only direct route to create them. Hu et al. (2017) knocked out CsWIP1 with an optimised CRISPR/Cas9 system and an improved transformation protocol and recovered non-transgenic gynoeceous plants; the same platform also edited CsVFB1,

CsMLO8 and CsGAD1. Base or prime editing of CsACS genes could further stabilise femaleness.

Parthenocarpy. Parthenocarpy is essential for pollinator-free greenhouses. In cucumber it is a quantitative trait linked to higher auxin and gibberellin levels. A cucumber-specific CRISPR knockout for this trait is still emerging, but the approach is validated in tomato, where editing of SlIAA9 (Ueta et al., 2017) and SlAGL6 (Klap et al., 2017) produced parthenocarpic fruit. Editing the conserved cucumber orthologues is a rational route to heritable parthenocarpy in greenhouse parental lines.

Disease resistance. Disease resistance is the most advanced area of cucumber editing, mainly through the S-gene approach. Powdery mildew (*Podosphaera xanthii*) requires host MLO genes: CRISPR/Cas9 mutation of CsaMLO8 gives strong penetration resistance, while CsaMLO1 and CsaMLO11 add post-invasive defence (Tek et al., 2022). Shnaider et al. (2023) disrupted CsaMLO8 in the commercial cultivar 'Ilan' and obtained transgene-free, highly resistant lines. Knockout of eIF4E gave non-transgenic cucumber resistant to CVYV, ZYMV and PRSV-W (Chandrasekaran et al., 2016) and was extended to WMV, ZYMV and PRSV for large-scale production (Fidan et al., 2023). For downy mildew (*Pseudoperonospora cubensis*), candidate S-genes such as DMR6, validated in other crops, are promising targets.

Fruit quality. Fruit bitterness is caused by cucurbitacin C, synthesised by a nine-gene cluster. Shang et al. (2014) showed that the transcription factors Bt and Bi regulate this pathway in leaves and fruit, respectively, through the gene Bi. Precise editing of Bt can guarantee non-bitter fruit while keeping the pest-deterrent bitterness of the leaves. Fruit firmness, skin colour, spines and shelf life are further targets once their loci are validated.

Table 2. Editable trait targets for greenhouse cucumber hybrids, with the genes, editing approaches and supporting studies discussed in the text. QTL, quantitative trait locus.

Trait category	Target gene(s)	Editing approach and outcome	Status in cucumber	Reference(s)
Gynoecey / sex expression	CsWIP1; CsACS1G / CsACS	Cas9 knockout of CsWIP1 → stable gynoecey; dosage tuning of CsACS by base/prime editors or dCas	Demonstrated (transgene-free)	Hu et al., 2017
Parthenocarpy	SlIAA9, SlAGL6 (orthologues); auxin-GA pathway	Editing auxin-signalling and MADS-box targets → fruit set without fertilisation	Validated in tomato; transferable	Ueta et al., 2017; Klap et al., 2017
Powdery-mildew resistance	CsaMLO8, CsaMLO1, CsaMLO11	Cas9 loss-of-function of MLO S-genes → durable recessive resistance	Demonstrated (transgene-free lines)	Tek et al., 2022; Shnaider et al., 2023
Virus (potyvirus) resistance	eIF4E	Cas9 knockout of eIF4E → broad resistance (ZYMV, PRSV, WMV, CVYV)	Demonstrated (transgene-free)	Chandrasekaran et al., 2016; Fidan et al., 2023
Downy-mildew resistance	DMR6 (candidate); QTL	Editing of candidate S-genes, translated from other crops	Candidate / emerging	Reviewed in text
Non-bitter fruit quality	Bt, Bi (fruit); Bl (leaf)	Precise editing of Bt / Bi → non-bitter fruit while bitterness is retained in the foliage	Pathway resolved; strategy defined	Shang et al., 2014

Next-Generation Editing for Precision Cucumber Improvement

Cas9 knockouts suit loss-of-function goals such as S-gene resistance and non-bitterness, but other objectives require a precise change of sequence or expression. Base and prime editing can create specific resistant alleles or fine-tuned promoter and coding variants without destroying the gene (Anzalone et al., 2019). The crRNA array of Cas12a (Zetsche et al., 2015) can edit, for example, an MLO gene, eIF4E and a sex or quality gene in one event, while CRISPRa and CRISPRi give graded regulation of CsACS or cucurbitacin genes. Together, these tools move cucumber improvement from single knockouts to precise, multi-trait engineering.

Integrating Gene Editing into Hybrid Seed Production

Commercial cucumber is sold as F1 hybrids, so the most direct strategy is to edit elite inbred parents, introducing resistance, sex, parthenocarp or quality alleles in the adapted background. Transgene-free edited lines differ from their progenitors only at the target locus and can enter existing hybrid combinations unchanged (Chandrasekaran et al., 2016; Hu et al., 2017; Shnaider et al., 2023). Speed breeding shortens the generations needed to remove the editing construct and fix edited alleles, and genomics-assisted selection tracks edited and background loci. The resulting workflow is shown in Figure 1.

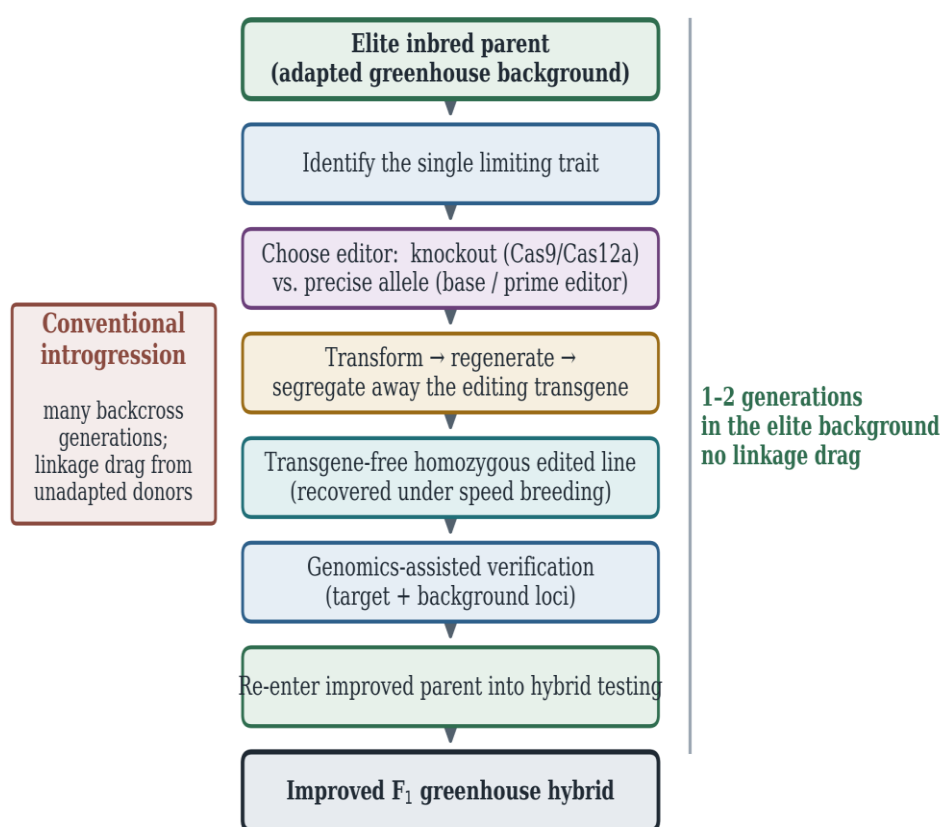


Figure 1. A pragmatic workflow for integrating gene editing into greenhouse cucumber hybrid seed production, contrasted with the many-generation route of conventional introgression

Challenges and Constraints. The main bottleneck is the low and genotype-dependent efficiency of *Agrobacterium*-mediated transformation and *in vitro* regeneration in cucumber, so genotype-flexible and tissue-culture-free delivery is a priority. Off-target effects must still be checked, although high-fidelity nucleases and careful guide design reduce the risk. The efficiency of prime editing also remains low. Finally, regulation differs between countries: some treat transgene-free edited plants like conventional varieties, others as genetically modified organisms, which affects the route to market from the start of any programme.

Future Perspectives. Base and prime editing will shift cucumber breeding from knockouts to the precise creation of alleles, and multiplexing with Cas12a will make multi-trait stacking routine. Combining editing with high-throughput phenotyping, pan-genome resources and genomic prediction will speed up the discovery of causal loci. Progress will depend on better transformation methods and on science-based regulation.

Conclusions

CRISPR/Cas9 and its next-generation derivatives form a practical toolkit for greenhouse cucumber hybrids. Published studies already cover the core traits: gynocery (CsWIP1), powdery-mildew resistance (CsaMLO8 and related MLO genes), virus resistance (eIF4E) and non-bitter fruit (Bi/BI/Bt), with parthenocarp supported by evidence from tomato. Base editing, prime editing, multiplexed Cas12a and dCas regulation will turn single-trait knockouts into precise multi-trait engineering. Once transformation efficiency and regulatory clarity improve, genome editing combined with speed breeding and genomics-assisted selection can markedly shorten the development of high-yielding, disease-resistant, high-quality greenhouse cucumber hybrids.

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