

REVIEW

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Advances and Future Perspectives of Targeted Mutagenesis in Barley

Hiroshi Hisano*

Institute of Plant Science and Resources, Okayama University, Chuo 2-20-1, Kurashiki, Okayama 710-0046, Japan

* Corresponding author: hiroshi.hisano@okayama-u.ac.jp | ORCID: 0000-0001-8457-253X

Abstract

Targeted mutagenesis technologies, commonly referred to as “genome editing”, have rapidly become transformative tools in crop breeding. Modern crop production faces mounting challenges such as climate change, emerging diseases, and shifting quality demands, creating an urgent need for breeding approaches that enable more precise genetic improvement within shorter timeframes. Targeted mutagenesis capable of introducing mutations accurately and efficiently began with the development of meganucleases and subsequently evolved through ZFNs, TALENs, and CRISPR/Cas systems. Recent technological advances, including multiplex editing and DNA-free genome editing, have made direct modification of elite cultivars increasingly feasible, helping to overcome long-standing barriers associated with genotype dependency and the complexity of polyploid genomes. Targeted mutagenesis has now been applied to a wide range of breeding targets, including disease resistance, tolerance to abiotic stresses, yield-related traits, and quality traits. Moreover, machine learning and AI are beginning to support target selection and phenotype prediction, ushering in an era in which genome editing can be integrated into breeding programs more rationally and efficiently. These scientific developments coincide with regulatory shifts in many countries toward easing restrictions on new genomic techniques (NGTs), particularly by treating SDN-1 genome-edited crops equivalently to those produced through conventional breeding. Such changes are expected to accelerate field trials, commercialization, and international collaboration. Nevertheless, several challenges remain, including bottlenecks in transformation efficiency, off-target assessment, the control of complex traits, societal acceptance, and unresolved regulatory details. Even so, genome editing is poised to become a central technology in next-generation cereal breeding, enabling the development of cultivars with greater resilience, productivity, and adaptability to diverse industrial needs.

Keywords: CRISPR/Cas9; genome editing; *Hordeum vulgare*; plant breeding; transformation amenability

1. Introduction

Barley (*Hordeum vulgare*) is one of the major cereal crops worldwide, following wheat (*Triticum aestivum*), maize (*Zea mays*), and rice (*Oryza sativa*). It serves diverse purposes, including food, feed, and malting, and contributes approximately 150 million tons to annual global production, forming a key component of agricultural systems across many regions [1]. However, climate change, emerging diseases, soil degradation, and shifting demands from farmers and consumers continue to drive the

need for new cultivars. These challenges underscore the necessity for breeding methods that enable more rapid and precise improvement than conventional approaches.

Conventional breeding has achieved substantial progress in yield, disease resistance, and quality traits, yet it also faces significant limitations. A major bottleneck is the depletion of accessible genetic resources. When available germplasm lacks desirable traits, breeders may need to rely on wide crosses with landraces or wild relatives, which substantially prolong breeding timelines. Moreover, introducing traits from genetically diverse wild relatives often results in linkage drag, necessitating numerous backcrosses to recover elite genetic backgrounds.

Targeted mutagenesis, commonly referred to as genome editing, has emerged as an innovative approach to overcome these constraints. Because specific genes can be precisely modified, elite cultivars can be directly improved, representing a major advantage over conventional methods. In polyploid crops such as wheat, simultaneous targeting of homoeologous genes across the A, B, and D subgenomes can generate strong phenotypic effects. This review provides a comprehensive overview of recent advances in genome editing in barley, its applications in cultivar development, and current regulatory trends, and discusses prospects and remaining challenges.

From a breeding perspective, the greatest advantage of genome editing lies in its precision. Because specific alleles can be modified without altering the broader genetic background, resistance can be introduced directly into elite cultivars. This is particularly valuable for avoiding linkage drag associated with introgression from wild relatives.

2. Evolution of Targeted Mutagenesis Tools

Targeted mutagenesis began with the development of early meganucleases, followed by zinc finger nucleases (ZFNs), which represented the first generation of genome editing tools applied in plants and other organisms [2]. However, these tools required complex protein engineering and design, limiting their widespread adoption in plant research. The subsequent emergence of transcription activator-like effector nucleases (TALENs), together with the establishment of modular assembly systems, made genome editing more accessible, yet the labor-intensive design process continued to restrict their use to a limited number of plant species [3]. The field then advanced dramatically with the introduction of clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) systems. For detailed mechanisms of each tool, readers are referred to Hisano et al. (2021) [4]. As widely recognized, the versatility, simplicity, and precision of CRISPR/Cas9 technologies are remarkable, leading to the awarding of the 2020 Nobel Prize in Chemistry to Dr. Emmanuelle Charpentier and Dr. Jennifer Doudna. Over the past decade, rapid technological progress has been made in CRISPR/Cas9 optimization, discovery of new Cas proteins, multiplex editing, base editing, prime editing, and DNA-free editing [5]. In addition, machine-learning-based prediction models for mutation outcomes have been developed, improving the accuracy of target selection [6]. Meanwhile, TALENs continue to be used for organellar genome editing in plants [7,8].

Although Cas9 remains the most widely used nuclease, Cas12a (Cpf1) and Cas3 systems have also been developed for plant applications [9,10]. CRISPR/Cas9 is the predominant tool in cereals due to its flexible guide RNA (gRNA) design and high activity. Cas12a, which recognizes T-rich protospacer adjacent motifs (PAMs) and generates staggered DNA breaks, is well suited for knock-in editing [11,12]. Cas12a also processes polycistronic gRNAs autonomously, providing a major advantage in polyploid crops where simultaneous editing of multiple homoeologs is required [13]. Cas3, an advanced genome editing technology originating from Japan, enables extensive DNA deletions with exceptionally high specificity [14]. Base editing systems (CBE and ABE) utilize Cas9-derived nickases [15] and allow nucleotide substitutions without inducing double-strand breaks, making them useful for fine-tuning gene function or recreating natural variants. Prime editing, which fuses Cas9 to a reverse transcriptase and uses a gRNA as a template for cDNA synthesis, enables precise insertions, deletions, and substitutions [16].

For plant applications, efforts have also focused on optimizing genome editing components and identifying suitable targets. For example, RNA polymerase III (Pol III) promoters isolated from the same species have been used to enhance gRNA expression, and systems for more stable Cas9 expression, through promoter selection or codon optimization, have been established [17,18]. Multiplex editing has become an efficient strategy in crop breeding. Many quality traits are controlled by multiple gene families, making simultaneous editing of several loci advantageous. Multiplex editing can be achieved either by introducing multiple gRNA expression cassettes or by transcribing a single polycistronic mRNA that is subsequently processed into individual gRNAs [19,20].

Targeted mutagenesis without introducing exogenous DNA has gained importance from regulatory and societal perspectives. DNA-free editing using CRISPR/Cas ribonucleoproteins (RNPs) has been successfully demonstrated in crops and may fall under more permissive regulatory categories [21,22]. In plant species amenable to virus vectors, transient expression followed by segregation can also be used to eliminate transgenes [23]. These approaches reduce off-target risks associated with prolonged nuclease expression and facilitate regulatory compliance. Numerous computational tools have been developed for target and off-target prediction [24].

3. Status of Targeted Mutagenesis in Barley

3.1 Development of Efficient Transformation Systems

Efficient transformation systems are essential for applying targeted mutagenesis in barley. Historically, barley has been considered recalcitrant to transformation, and only a limited number of cultivars were amenable to genetic modification, slowing the integration of targeted mutagenesis into breeding programs. Barley transformation has most relied on the cultivar 'Golden Promise', which exhibits high susceptibility to *Agrobacterium*-mediated transformation and has enabled numerous gene function studies [25]. In cultivars less amenable to *Agrobacterium*-mediated transformation, particle bombardment is used, although it often results in multicopy insertions or fragmented endogenous DNA. Recent advances, including optimized tissue culture conditions and identification of loci associated with transformation efficiency, have expanded the range of transformable barley cultivars [26-28]. Moreover, introducing morphogenic regulators such as *WUSCHEL2* (*WUS2*) and *BABY BOOM* (*BBM*) has dramatically improved regeneration efficiency and reduced genotype dependency in barley [29]. DNA-free editing using CRISPR/Cas ribonucleoproteins (RNPs) has also been demonstrated in plants, enabling the generation of edited lines without exogenous DNA [30].

3.2 Biotic and Abiotic Stress Tolerance

The first successful examples of genome editing in barley were reported by Wendt et al. (2013) and Lawrenson et al. (2015) for TALENs and the CRISPR system, respectively, experimentally demonstrating that these tools can be effectively used in barley [31,32].

Disease resistance represents one of the most active areas of targeted mutagenesis in barley, driven by the need to reduce fungicide use and stabilize yields under environmental fluctuations. Early landmark studies demonstrated that CRISPR/Cas9 systems can efficiently disrupt susceptibility genes and confer durable resistance without introducing foreign DNA. In hexaploid wheat, ahead of barley, simultaneous editing of the three subgenome alleles of the MILDEW-RESISTANCE LOCUS (*TaMLO*) conferred strong, genetically stable resistance to powdery mildew, demonstrating the feasibility of multi-allelic editing in polyploid crops [33]. In barley, *HvMLO* knockout lines have similarly contributed to powdery mildew resistance research [34]. A double knockout of two members of the mitochondrial *HvMORC* gene family successfully generated barley lines exhibiting resistance to both *Blumeria graminis* and *Fusarium graminearum* [35]. Targeted mutagenesis of *NFXL1*, a gene associated with fusarium head blight, has generated novel resistance alleles in wheat; however, no comparable resistance genes have yet been identified in barley [36]. Kis et al. (2019) also reported successful

generation of wheat lines exhibiting strong resistance to wheat dwarf virus, demonstrating the feasibility of conferring antiviral resistance through genome engineering [37].

Seed dormancy and pre-harvest sprouting (PHS) are critical traits affecting grain quality and storability in barley. Genome editing has contributed to dissecting and modifying the dormancy regulatory network, including ABA signaling and key transcription factors. In malting barley, achieving an optimal balance between dormancy and germination is essential. *HvQsd1* and *HvQsd2* have been identified as central dormancy genes, and CRISPR/Cas9-based targeted mutagenesis has demonstrated the ability to precisely adjust dormancy strength and germination timing [38]. Taken together, these studies demonstrate that targeted mutagenesis has substantially advanced both the understanding and improvement of stress tolerance in barley, establishing it as a practical and increasingly indispensable tool for breeding resilient cultivars.

3.3 Yield, Plant Architecture, and Quality Traits

Yield-related traits are governed by complex genetic networks, making them sometimes tough to improve through conventional breeding. Targeted mutagenesis of the *HvGA2ox8a* gene increases plant height, enabling enhanced biomass production [39]. Improved lodging resistance has also been achieved by knocking out gibberellin 20-oxidase2 (*GA20ox2*) using CRISPR/Cas9 in barley [40]. Karunarathne et al. (2022) reported that a mutation in the abnormal cytokinin response1 repressor 1 (*HvARE1*) gene led to improved nitrogen use efficiency [41].

Quality traits are also important targets. In wheat, CRISPR-based mutagenesis of α -gliadin gene families has produced “low-gluten” lines with reduced immunogenic epitopes associated with celiac disease [42-44]. In barley, editing starch biosynthesis genes has enabled modification of the amylose/amylopectin ratio, with potential applications in malting, distilling, and food processing [45,46]. Zeng et al. (2020) reported improved vitamin E biosynthesis through CRISPR/Cas9-mediated targeted mutagenesis of homogentisate geranylgeranyl transferase (*HGGT*) and homogentisate phytyltransferase (*HPT*) [47]. For improving biofuel production in barley, Lee et al. (2021) targeted the caffeic acid O-methyltransferase 1 (*HvCOMT1*) gene and demonstrated that its mutation reduces lignin content and increases sugar yield [48]. The authors’ research group successfully introduced targeted mutations in the lignin biosynthesis gene *HvCAD*, reducing lignin content and revealing pleiotropic effects such as the orange lemma phenotype [49]. Gu et al. (2022) targeted *SPDT*, a phosphorus transporter, altering phosphorus allocation and demonstrating the potential for developing low-fertilizer-input cultivars [50]. Additionally, introducing mutations in *NUD*, the gene controlling hull adhesion, enabled rapid development of “naked” barley lines suitable for malting in crosses between malting cultivars and ‘Golden Promise’ [51]. Collectively, these findings show that targeted mutagenesis is effective for improving diverse traits related to yield, plant architecture, and quality, thereby expanding the potential of barley breeding.

4. Future Trends, Challenges, and Perspectives in Targeted Mutagenesis for Crop Breeding

Countries such as Japan and the United States have taken early steps in establishing regulatory pathways for genome-edited crops, and several are already cultivated commercially as of 2025 [52]. In Japan, high-GABA tomatoes were sold on a trial basis in 2021, and by 2026, high-sugar tomatoes, waxy maize, small-tuber potatoes, and bananas with reduced browning are expected to become commercially available. In Europe, long-standing regulations that treated genome-edited crops as GMOs are being reconsidered. Under the emerging framework for new genomic techniques (NGTs), site-directed nuclease (SDN) 1 modifications may be regulated similarly to conventionally bred varieties [53,54]. This shift represents a major turning point for crop breeding. Note that SDN-2 and SDN-3 are regulated as recombinant DNA (genetically modified) plants. The principles underlying these methods are illustrated in Figure 1.

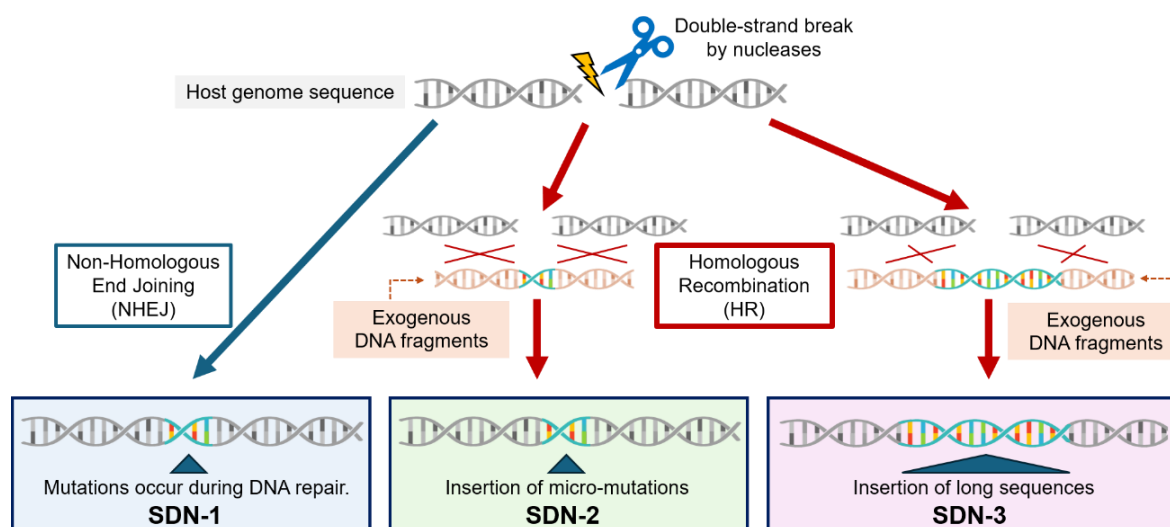


Figure 1. Types of Mutations Introduced by Site-Directed Nuclease (SDN) Techniques

SDN-1: In this approach, the target host genomic sequence is cleaved by a nuclease via non-homologous end joining (NHEJ), and small mutations (indicated by a triangle), such as short insertions, deletions, or substitutions, are introduced during the natural DNA repair process.

SDN-2: In this approach, after nuclease-mediated cleavage, homologous recombination (HR) occurs between the target genomic sequence and an exogenous DNA fragment homologous to the target sequence and containing a small, intended change. As a result, the mutation (indicated by a triangle) is introduced into the genome. Although the resulting mutation is indistinguishable from naturally occurring mutations, organisms produced by this method are regulated in the same manner as genetically modified (recombinant DNA) plants.

SDN-3: In this approach, after nuclease-mediated cleavage, HR occurs between the target genomic sequence and an exogenous DNA fragment homologous to the target sequence and containing a desired long DNA sequence. This allows the introduction of a long DNA sequence (indicated by a wide triangle) into the target genome. SDN-3 enables the precise insertion of complete genes or other genetic elements into a specified genomic region. Organisms produced by this method are treated as genetically modified (recombinant DNA) plants.

For barley, a key crop in European agriculture, regulatory relaxation is expected to accelerate the development and field testing of genome-edited lines targeting disease resistance, climate resilience, and quality traits. Reduced regulatory burden will allow breeders to evaluate agronomic performance, environmental responses, and trait stability more rapidly. This is particularly important for traits strongly influenced by field conditions, such as tolerance or resistance to heat, drought, pre-harvest sprouting, and disease.

Despite these positive developments, several challenges remain. Public perception and consumer acceptance continue to influence market adoption, especially in regions with strong skepticism toward biotechnology. Traceability and detection of SDN-1 edits pose regulatory and methodological difficulties, as such edits may be indistinguishable from natural mutations. Intellectual property issues—including patents related to CRISPR tools and edited alleles—may also limit access for small breeding programs and public institutions.

Off-target effects require careful evaluation, yet comprehensive analysis is challenging in large and complex genomes such as those of barley and wheat. Whole-genome sequencing of edited lines remains costly for large-scale breeding programs and ensuring genome stability across generations is



essential for regulatory approval and commercial deployment. Advances such as AI-assisted off-target prediction and high-precision editing systems are expected to mitigate these issues.

5. Conclusion

Targeted mutagenesis is poised to become a core technology in next-generation cereal breeding, enabling the development of cultivars with enhanced resilience, productivity, and suitability for diverse industrial applications. Because targeted mutagenesis can be applied directly to elite cultivars, the need for extensive backcrossing is greatly reduced, thereby shortening the breeding cycle. As transformation technologies, editing toolkits, and regulatory frameworks continue to advance, the integration of genome editing into commercial breeding programs is expected to accelerate further in the coming years.

Data Availability Statement

No new data was generated.

Author Contributions

Conceptualization, Investigation, and Writing: H.H.

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Ethics approval and consent

Not applicable.

Competing Interests

The author declares no competing interests.

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Author Information

Full Name	Hiroshi Hisano
Department	
Institution	Institute of Plant Science and Resources, Okayama University, Chuo 2-20-1, Kurashiki, Okayama 710-0046, Japan
Email	hiroshi.hisano@okayama-u.ac.jp
ORCID	0000-0001-8457-253X
Scopus ID	