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Review Article

Status and contribution of CRISPR/Cas9 based gene-editing system in the development of a low-immunogenic wheat variety

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Abstract

Gluten, a wheat protein, contains epitopes that trigger celiac disease (CD). So far, there is no treatment available for CD other than following a life-long, strict gluten-free diet (GFD). A very low-gluten or gluten-free wheat could provide an alternative treatment to CD. Till date, conventional plant breeding methods are not sufficient to produce celiac-safe wheat. Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9 (CRISPR/Cas9) is a versatile gene-editing system may efficiently edit the immunogenic gluten protein thereby producing a celiac-safe wheat variety. However, CRISPR/Cas9-mediated genome editing system has not been widely investigated to edit the wheat genome. Published literature available on various scientific platforms, that applied the CRISPR/Cas9 system to edit the wheat genome were explored. Only original research articles were included. Review articles, protocols, scientific presentations, and Ph.D. thesis were excluded. CRISPR/Cas9 is a highly specific gene-editing technology that can be used to efficiently edit the complex hexaploid wheat genome. It targets a specific wheat genome locus, delete an immunogenic gene and replace it with a preferred gene. CRISPR/Cas9 technology could be a breakthrough in providing an alternative treatment for CD. However, further studies are required to efficiently apply this gene-editing technology to develop a celiac-safe wheat variety.

Keywords: CRISPR/Cas9; celiac disease; wheat; sgRNA; gluten; low-immunogenic wheat

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Introduction

Common wheat (*Triticum aestivum)* is one of the world's most preferred staple food [1]. However, ingestion of wheat in susceptible individuals may trigger several food-related complications collectively known as Gluten-related disorders (GRD) [2]. The most prevalent and extensively studied GRD is celiac disease (CD) [2]. It is a T-cell mediated chronic enteropathy caused by the ingestion of immune-dominant gluten peptides in genetically predisposed individuals who possess specific Human Leukocytic antigen (HLA)-DQ2 and/or HLA-DQ8 alleles [2,3]. CD affects almost 1-1.6% population all over the globe [4]. Complete avoidance of gluten from diet is the only accepted treatment available for CD and other GRDs, namely wheat allergy (WA), dermatitis herpetiformis (DH) and Non-Celiac Gluten Sensitivity (NCGS) [2,3,5]. Gluten is a ubiquitous protein that is universally used not only in cereal-based products but also in numerous other commercially prepared food and non-food items [6]. Therefore, it is difficult to eliminate gluten completely from the diet [6,7].

Gluten (storage protein of wheat) contains Gliadins and glutenins. While gliadin makes the dough soft, glutenins provide a fine baking quality to wheat [8]. Gliadin is sub-divided into α-, γ-, and ω-subfractions, out of which α-gliadin contains critical epitopes responsible for CD development [9]. There are two fractions of glutenins i.e., low molecular weight (LMW) and high molecular weight (HMW) glutenins [8]. Gliadin is encoded by multiple gene families that are arrayed on specific loci in a repetitive sequence fashion and are located at *Gli-2* loci on chromosome 6A, B and D [2,9]. α-gliadin contains the 33-mer peptide, particularly rich of proline-glutamine sequences. Human intestinal and pancreatic enzymes are unable to completely digest the complex amino acid sequence of α-gliadin which is partially broken down into larger peptides [2,10]. These peptides pass through intercellular junctions and enter

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the lamina propria where tissue transglutaminase enzyme deamidates this gluten fraction that is recognized by HLA-DQ heterodimers attached to antigen presentation cells (APC). The HLA-gluten complex then triggers T-cells to induce the pro-inflammatory cascade which eventually leads to CD [10].

Gluten-containing cereals, particularly wheat, is used extensively by the food industry mostly to prepare bread and pasta [11]. Wheat was introduced in the human diet about 10,000-12,000 years ago[12]. The first domesticated wheat varieties were diploid and tetraploid. Einkorn wheat had only one genome i.e. the A genome (diploid). This wheat variety was designated as *Triticum monococcum* and had less protein content in comparison to modern wheat. This wheat variety is scarcely consumed by humans nowadays [12].

The tetraploid wheat domesticated simultaneously with diploid wheat, contains two genomes (AA & BB) hence it is termed as tetraploid wheat. However, the A-genome is similar to *Triticum monococcum and the* B-genome is similar to *Triticum speltoides.* Durum wheat (*Triticum durum or Triticum turgidum*) is a tetraploid species of wheat that is used to prepare pasta mostly[12]. It is reported that diploid or tetraploid wheat (ancient wheat varieties) with AA/BB genome were less-immunogenic to CD patients in comparison to the recent wheat verities [12,13].

Currently used bread wheat/common wheat (*Triticum aestivum*) is an allohexaploid species with three genomes (AA, BB, DD) resulting from natural hybridization between a tetraploid *T. turgidum* (*dicoccum*) carrying the AA, BB-genome, and the wild diploid species *Triticum tauschii* carrying the DD-genome [14]. While the introduction of the D-genome improved the bread-making properties, most of the immunogenic peptides in CD are coded by the D-genome [12]. α-gliadin, encoded on D-genome is more immunogenic and recognized by T-

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cells easily. There are preliminary evidence that primitive wheat (diploid or tetraploid) is safer and less immunogenic compared to currently used hexaploid wheat as ancient wheat varieties had less immune-dominant protein fractions [12,13]. Wheat variety with low T-cell stimulatory epitopes may reduce the chances of developing CD. Exposure to improved wheat variety with low-immunogenic wheat may not cause an intense immunological trigger to CD patients hence it could be useful for CD management [8,15].

Multiple efforts have been imposed to develop a safer variety of wheat through the combination of conventional sophisticated tools (mutation, breeding) however, till date they are not fully successful in developing a celiac-safe wheat variety. Most of such studies involved analytical techniques and/or RNA interference (RNAi) to produce a wheat kernel with a reduced percentage of immunological peptides (α-gliadin). However, a safer variety of wheat has not been developed so far [8,15–18].

In recent years, gene editing techniques (ZFN, TALEN) emerged as a promising approach to edit or delete the toxic gluten fractions in wheat [19]. CRISPR/Cas9 is a novel second-generation genome editing system. This technique can apply in wheat to efficiently modify qualitatively and/or quantitatively the gliadin content in wheat grains [9]. CRISPR/cas9 system targets a particular genome locus and delete a desired gene within the genome, this machinery may use in the wheat to replace the toxic gluten gene with a preferred low-immunogenic gene to produce gluten-free/low gluten-containing wheat. This modified low-gluten or gluten-free wheat will be a safe option for CD and GRD patients [8]. However, so far less is known about the application of CRISPR in wheat genome modification and there is a lot to explore in the coming future. There are few studies available which have reported the implication of CRISPR/Cas9 techniques to produce a low-immunogenic/gluten-free wheat. To

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the best of our knowledge, this review is among the first articles which provides an overview of the current status and contribution of CRISPR/Cas9 application in the editing of the wheat genome.

Objectives and Research Question

The aim of this review article was to explore the current status and contribution of CRISPR-Cas9 tool in improving the wheat genome to develop a celiac safe wheat variety.

Literature review

Published literature related to the application of CRISPR/Cas9 in developing celiac-safe wheat variety were searched using the keywords 'celiac and CRISPR', 'CRISPR in celiac disease', 'Wheat engineering with CRISPR', 'Low immunogenic wheat and CRISPR', on the electronic databases like PubMed, Google Scholar, CrossRef, CiteFactor, Academic Resource Index, Research Gate, and WorldCat. We also searched for references from the published articles. No publication date was imposed. Only original articles that applied CRISPR/Cas9 for gene editing in wheat crops were included. Review articles, protocols, scientific presentations, and Ph.D. thesis were excluded. Following these criteria, in total 56 studies were explored. Of them, 14 articles were selected and 10 articles were found appropriate.

What is the gene-editing technique?

Gene editing or site-directed mutagenesis include a range of molecular techniques that allow the introduction of specific alteration in the genome of a cell or organism. These alterations can include removing a specific sequence of the genome, adding genes at a specific location

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or substituting DNA sequences at the desired location in the genome [20]. The main concept behind the genome editing techniques is to employ engineered endonucleases to make site-specific DNA Double-Strand Break (DSB), which triggers the cellular intrinsic DSB repair mechanism to repair the breaks [21]. These repairs can be tricked to add, remove, or substitute a series of nucleotides in the genetic code, thus enabling the introduction of known desired alleles in the target organism [22]. The major DNA DSB repair mechanisms include Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) [19,23]. In the NHEJ repair pathway, the two broken ends are joined with a loss of few nucleotides hence it is a mutation prone process and taken advantage in knocking out of gene function in gene editing [24]. HR is a highly precise repair mechanism and exploited in genome editing techniques to edit single nucleotides or to introduce other precise changes in the genome [25]. Overall the vital component of these gene-editing techniques is chimeric endonuclease (engineered endonucleases) [8,19,22]. There are different families of engineered endonucleases used for the digestion of the desired sequence of nucleotides. These are divided into the first generation genome editing tool i.e. Mega-nucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector-based Nucleases (TALEN), and the second-generation gene-editing tool that includes the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) system [19,26]. Among all these, CRISPR/cas9 is the latest gene-editing tool that is highly accurate, rapid, simple and comparatively cheaper than other gene-editing tools [27,28]. CRISPR/Cas9 system is successfully applied for plant genome improvement and in various human diseases such as gastrointestinal, hematologic, viral, and cancer [9,29].

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What is CRISPR-Cas9?

The concept of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas9 (CRISPR-associated protein 9) was adopted from the defence machinery of bacteria [19,25,28]. When a virus (Bacteriophage) attacks a bacteria, the bacteria captures snippets of the genetic material of the virus (DNA) and synthesize DNA segments that are known as CRISPR arrays [19,25,26]. These CRISPR arrays memorize the virus, on future invasions of the same or similar virus, the bacteria synthesize the RNA segments from the CRISPR arrays that target the virus. The bacteria use Cas-9 enzyme to cleave the targeted DNA sequence that eventually neutralizes the virus [28].

There are two main components of CRISPR, first one is single guide RNA (sgRNA) which is complementary to the target sequence and the second one is the Cas-9 gene which is adapted from *Streptococcus pyogenes* (SpCas9) that requires a G-rich (5'-NGG-3') PAM (protospacer-adjacent motif) site [28,30,31].

sgRNA is a small sequence of nucleotides (18-21 nucleotides), complementary to the target DNA, with three PAM sites at 3' end followed by RNA scaffold [32]. Cas9 protein comprise of two functional domains, the first one is large recognition (REC) and the second one is smaller nuclease (NUC) domain. The NUC domain has two conserved endonuclease sites (RuvC and HNH) and a PAM interacting site. RuvC cleaves the non-complementary strand while HNH cleaves the complementary sequence to sgRNA [8,32,33].

The in-vivo gene-editing mechanism can be employed successfully in in-vitro conditions. In the laboratory a small RNA strand (sgRNA), specific to the target DNA, is synthesized, this sgRNA is attached with the Cas9 (nuclease). The sgRNA-Cas9 complex is then introduced

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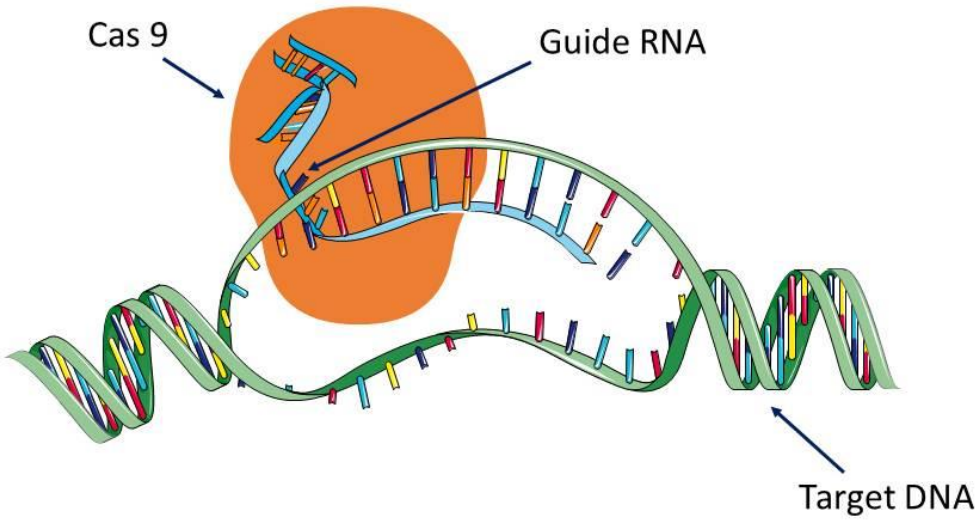
into the target cell. Within the target cell, the sgRNA identifies and binds with the complementary sequence on the DNA of the cell. Thereafter, the Cas9 cuts the particular location of DNA (Figure 1) [19,25,28]. After the cleavage of the DNA sequence, the cell's own DNA repair machinery (NHEJ or HR repair mechanisms) starts to repair the break. This mechanism can be used to add or delete DNA sequences and also replace a segment of DNA from a customized DNA sequence [32–34].

Figure 1: Schematic diagram showing sgRNA-guided DNA binding of Cas9. The diagram illustrates that a sgRNA specifically targets and bind to a short DNA sequence tag (the PAM), unzips complementary DNA to create a sgRNA–target DNA heteroduplex.



PAM:protospacer-adjacent motif; sgRNA:single guide RNA.

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CRISPR/Cas9 in wheat genome editing

The CRISPR/Cas9 tool has been successfully applied in various crops to improve their traits [35]. However, it is challenging to apply this technique in the wheat crop as wheat has a very complex genome. The wheat genome is a long 17 giga-bases genome with a high content of repetitive sequences that make it hard to insert target mutants. Apart from this, the modern wheat is an allohexaploid with three homologous copies of most of the genes in the genome A, B, and D. Targeting multiple copies of a gene is challenging for gene editing [8,19]. However, the recently improved sgRNA, due to the orthologues of the Cas9 gene, is capable to target multiple genes simultaneously [9,35]. In recent years, researchers have applied the CRISPR/Cas9 in modern wheat to produce celiac-safe wheat [8,27,36]. CRISPR/Cas9 is validated in different grains like rice, tomato, barley however, it is still not fully applied in wheat [8,16,19]. Nevertheless, lately several research groups have targeted multiple wheat-genome loci to display abiotic and biotic stresses in wheat [9,27,32,33]. Most targeted wheat loci were TaMLO, TaMLO-A1, TaLOX2, PDS, INOX, TaGW2 [14,30,37]. The recent development of CRISPR tool in wheat has been summarized in 错误!未找到引用源。.

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Table 1: Summary of the studies applied CRISPR/Cas9 system to edit Wheat genome**.**

S.No.	Author	Year	Targeted wheat genes	Conclusion
1.	Jouanin A. et al.[9]	2019	Six sgRNA sequences (sgRNA-α87, α213, α324, γ86, γ272, γ603) were designed to target α-/γ-gliadins or both simultaneously	CRISPR/Cas9 can edit multiple genes simultaneously
2.	Zhang S. et al.[38]	2019	Five wheat genes (TaPinb, TaDA1, TaDA2, TaNCED1, TaLPR2) were targeted via Agrobacterium-mediated transformation of an sgRNA	Agrobaterium-mediated CRISPR/Cas9 system is a useful method to improve wheat genome
3.	Sánchez-León S. et al.[8]	2018	α- gliadin genes were targeted by two sgRNAs (sgAlpha-1 and sgAlpha-2)	Generated a low-gluten, transgene-free wheat lines
4.	Zhang S. et al.[31]	2018	Three genes (Pinb, waxy and DA1)) of common wheat (*Triticum aestivum L.*) were targeted through CRISPR/Cas9 delivered via Agrobacterium tumefaciens.	Agrobacterium-mediated CRISPR/Cas9 system can facilitate wheat genome editing
5.	Kim D. et al.[36]	2018	Targeted two wheat genes (TaDREB2 and TaERF3)	CRISPR/Cas9 has huge potential for the manipulation of wheat genome
6.	Bhowmik P.	2018	TaLox2 and TaUbiL1 genes were	CRISPR/cas9 is an efficient

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	et al. [39]		targeted by CRISPR/cas9 system combined with microspore technology	system to edit wheat genome and for trait discovery
7.	Wang W. et al. [35]	2018	TaGW2, TaLpx-1, and TaMLO genes were targeted by CRISPR-Cas9-based multiplexed gene editing	CRISPR-Cas9-based multiplexed gene editing system successfully manipulate wheat genome
8.	Liang Z. et al. [27]	2017	Wheat gene (TaGW2) was targeted by using gw2-sgRNA by CRISPR/Cas 9 ribonucleoproteins (RNPs) system	CRISPR/Cas9 ribonucleoproteins (RNPs) is efficient to produce genetically improved crops
9.	Wang Y. et al. [40]	2014	TaMLO-A1 gene	CRISPR/Cas9 can improve bread wheat genome
10.	Upadhyay S.K. et al.[37]	2013	INOX and PDS genes	CRISPR/Cas9 is a powerful method for wheat genome engineering

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Discussion:

To date, only accepted treatment of CD is a life-long complete exclusion of gluten from the diet and adherence to a true GFD [5]. There is a 100% positive effect of GFD on CD patients [41]. However, as gluten is a ubiquitous molecule that is used in almost every industrially-prepared food as well as numerous non-food products, it is almost impossible to exclude gluten from the diet [6, 7]. Moreover, exclusion of gluten from the diet of CD patients reduce the quality-of-life. Rigorous efforts have been carried out to explore an alternative treatment for CD which allows the wheat consumption in CD patients [10]. However, it is not possible so far to treat the CD. A possible and effective alternate treatment could be the use of a special variety of wheat that does not contain T-cell stimulatory epitopes. This option of treatment is possible by developing a "low-gluten/gluten-free" wheat variety that does not contain toxic peptides but maintains the basic properties of wheat [10,42,43]. In this regard, Molberg *et al.* extracted gluten from ancient wheat varieties and investigated T-cell stimulatory epitopes. The authors found different intestinal T-cell responses to the diploid wheat. The authors also confirmed that the immuno-dominant fraction of wheat is encoded by α-gliadin genes on chromosome 6D, thus it is absent from diploid einkorn (AA) and even certain cultivars of the tetraploid (AABB) wheat. It supports that the ancient variety of wheat did not contain the toxic fragment of gliadin peptides [44]. In another study, Van den Broeck HC *et al.* compared the genetic diversity of gluten proteins based on the presence of an immune-dominant epitope (Gli-α9) and less toxic epitope (Gli-α20) in 36 modern European wheat and 50 landraces representing the wheat varieties grown during the last century. The authors found a higher amount of Gli-α9 and a lower amount of Gli-α20 epitopes in comparison to landraces [45]. The outcome of this study again validated that modern wheat variety contains a high amount

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of toxic immune-dominant gliadin fragments. This study also documented that apart from awareness and improved diagnostic techniques for CD, an increase in the wheat breeding has a possible contribution to the increasing prevalence of CD [45]. Multiple studies have supported this finding later [12,44,46,47]. Although these methods have confirmed that the ancient wheat varieties had low immunogenic properties, conventional wheat breeding methods are still struggling to develop a safe wheat variety for CD patients [8,15].

Gene editing techniques have shown favourable results in this regard. However, these methods have some limitations. In some studies, researchers have successfully silenced gliadin expressions using RNA interference (RNAi) [48,49]. However, the insertion of RNAi construct into the wheat genome comes under the process of genetically modified (GM) plants. Due to the high cost of the regulatory process of GM plants, it is difficult to use this process for developing commercial low-immunogenic wheat varieties. Moreover, GM plants are banned in many countries. Mutation breeding has also given a promising response but applying gamma-radiation to the polyploidy plant is tough and may cause DNA single/double-strand breaks, the repairing method Non-Homologous End Join (NHEJ) is an error-prone method and may lead to large base-pair deletion/ inversion. It may also remove whole gliadin gene or multiple genes [16–18].

In comparison to these techniques, CRISPR/Cas9 is a relatively easy and specific method to edit genes. In the last decades, multiple studies have been conducted to edit the wheat genome using CRISPR/Cas9 and have shown promising results to develop a low immunogenic wheat variety. However, modern wheat/bread wheat (*T. aestivum*) has a hexaploid genome which is difficult to target by other gene-editing techniques. CRISPR/Cas9 is an advanced and highly sophisticated and specific method that can target the complex

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genome of wheat to replace the toxic genes within the genome with the non-toxic genes in order to develop a non-toxic wheat variety. In this article to the best of our capabilities, we explored the published articles through various scientific dabase and on the basis of those, we have summarized the current status of CRISPR/cas9 system to edit the wheat genome. Still, there could be some articles that may have missed. However, our search showed that there are few studies so far that have applied CRISPER/Cas9 system in wheat genome and have shown promising results (Table 1) [8,9,30,35,37,38].

Shan Q *et al*. and Upadhyay SK et al. have knocked out the TaMLO, TaPDS, and TaINOX loci in order to validate CRISPR/cas9 in wheat [30,37]. In another study, Jouanin *et al.*, targeted six wheat genes separately or simultaneously. This study successfully proved that CRISPR/Cas9 can be used for editing multiple genes concurrently in polyploidy bread wheat [9]. Zhang S. *et al.* have targeted five genes in common wheat using the CRISPR/Cas9 system delivered via Agrobacterium tumefaciens and showed that Agrobacterium-mediated CRISPR/Cas9 system can be used for targeted mutations and facilitated wheat genome improvement [38]. Sànchez-Leòn *et al.* have claimed to generate a low-gluten, transgene-free wheat line that could be used for developing safe wheat varieties and also for producing low-gluten containing food products. The authors have targeted a conserved region adjacent to the genetic sequence codes for the main immune-dominant peptide of α- gliadin by constructing two sgRNAs (sgAlpha-1 and sgAlpha-2). They generated 21 mutant lines, 15 from bread wheat (*Triticum aestivum L.*) and 6 from durum wheat and showed prominent reductions in the α- gliadins. Moreover, one of the lines showed a reduction in immunogenicity up to 85% [8]. In another study, Zhang *et al.* demonstrated that the Agrobacterium-mediated CRISPR/Cas9 system could be a useful method to improve the wheat genome. In this study,

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the authors have identified three genes (Pinb, waxy and DA1) in seven target sites to construct specific vectors and successfully applied targeted mutagenesis in wheat (*Triticum aestivum L*) protoplasm and transgenic T0 plant using CRISPR/Cas9 system delivered via *agrobacterium tumefaciens* [31]. Liang *et al.* introduced a new gene editing methodology for bread wheat using CRISPR/Cas9 ribonucleoproteins (RNPs). The authors showed that the RNP mediated CRISPR/Cas9 method has less chance of off-target mutations in wheat cell in comparison to conventional CRISPR/Cas9 technique as no foreign DNA is used in this method and the mutants obtained from this method are completely transgene free. The authors have claimed that this new method could be an appropriate choice to produce genetically improved crops [27]. Wang *et al.* have targeted three genes (TaGW2, TaLpx-1 and TaMLO) in hexaploid wheat by introducing CRISPR/cas9-based-multiplexed genome editing system [35].

Available evidence has shown that the CRISPR/Cas9 technique can be used to produce a less immunogenic and better variety of wheat. By improvising the current intricacy in methodology, it is possible to develop a safe variety of wheat for CD patients. If such gluten-free wheat restores its natural taste it would be easier for the CD patients to adhere completely to the GFD. About 50% of CD patients do not follow strict GFD because of multiple reasons including non-availability of gluten-free food, an appalling taste of GFD etc [50]. This is a challenging condition for CD management. Safe wheat variety for CD patients will eradicate this problem and CRISPR/Cas9 technology has the potential to produce such a variety of wheat

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Conclusions:

CRISPR/CAS9 system is a versatile gene-editing method that can precisely modify the conserved regions of the toxic fragment of gluten (α-gliadin) gene to produce a low-toxic or a completely safe variety of wheat for celiac disease patients. This method could be a major breakthrough for providing a promising alternative treatment for CD. However, the CRISPR/Cas9 method is currently not fully adapted in CD studies to modify the wheat genome. There is a need for multiple studies to apply this tool on different varieties of wheat and produce a celiac-safe wheat variety.

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