Review

Current Status and Perspectives on the Application of CRISPR/Cas9 Gene-Editing System to Develop a Low-Gluten, Non-Transgenic Wheat Variety

Anil K. Verma [1,*], Sayanti Mandal [2], Aadhya Tiwari [3,4], Chiara Monachesi [1], Giulia N. Catassi [5], Akash Srivastava [6], Simona Gatti [5], Elena Lionetti [5] and Carlo Catassi [5,7]

1 Celiac Disease Research Laboratory, Polytechnic University of Marche, Ancona, Italy; chiara.monachesi28@gmail.com
2 Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Ganeshkhind Road, Pune, Maharashtra 411007, India; mandalsayanti@gmail.com
3 Department of System Biology, MD Anderson Cancer Center, 77030, USA; ATiwari3@mdanderson.org
4 Laboratory of Cell Biology, Department of Orthopaedic Surgery, University Hospital of Tübingen, Waldhörnlestraße 22, D-72072 Tübingen, Germany
5 Division of Pediatrics, DISCO Department, Polytechnic University of Marche, Ancona, Italy; giulia.catassi@gmail.com (G.N.C.); simona.gatti@hotmail.it (S.G.); m.e.lionetti@univpm.it (E.L.); c.catassi@univpm.it (C.C.)
6 Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02906, USA; akashsriv1@gmail.com
7 Mucosal Immunology and Biology Research Center, Division of Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital, Boston, MA, USA
* Correspondence: anilkrvermaa@gmail.com or a.k.verma@pm.univpm.it; Tel.: +39-0715962834

Abstract: Wheat gluten contains epitopes that trigger celiac disease (CD). A life-long strict gluten-free diet is the only treatment accepted for CD. However, very low-gluten wheat may provide an alternative treatment to CD. Conventional plant breeding methods are not sufficient to produce celiac-safe wheat. RNA interference technology, to some extent, succeeded in the development of safer wheat varieties. However, these varieties had multiple challenges in their implementation. Clustered Regularly Interspaced Short Palindromic Repeats-associated nuclease 9 (CRISPR/Cas9) is a versatile gene-editing tool that has the ability to edit the immunogenic gluten genes. So far, only a few studies have applied CRISPR/Cas9 to modify the wheat genome. In this article, we reviewed published literature that applied CRISPR/Cas9 in wheat genome editing to investigate the current status of the CRISPR/Cas9 system to produce a low-immunogenic wheat variety. We found that in recent years, the CRISPR/Cas9 system has been continuously improved to edit the complex hexaploid wheat genome. Although some reduced immunogenic wheat varieties have been reported, CRISPR/Cas9 has still not been fully explored in editing the wheat genome. We conclude that further studies are required to apply the CRISPR/Cas9 gene-editing system efficiently for the development of celiac-safe wheat variety and to establish it as a "tool to celiac safe wheat."

Keywords: celiac disease; CRISPR/Cas9; RNAi; α-gliadin; low-gluten; non-transgenic wheat

1. Introduction

Common wheat (*Triticum aestivum*, $2n = 6x = 42$, AABBDD) is a preferred staple food worldwide [1]. During 2018/19, total global wheat consumption was 734.7 million metric tons which increased by 759 million metric tons during 2021 [2]. However, the consumption of gluten (a storage protein of wheat) in a huge number of susceptible individuals triggers several gluten-related disorders (GRDs) including celiac disease (CD) that affects 1–2% of the world population [3]. CD is a T-cell mediated chronic enteropathy caused by the ingestion of immuno-dominant gluten peptides in genetically predisposed

individuals who possess a specific human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8 genes [4–6]. Following a life-long strict gluten-free diet (GFD) is the only accepted treatment for CD [7]. Adherence to a strict GFD shows absolute regression in the celiac-associated symptoms (diarrhea, anemia, failure to thrive, weight loss, etc.) which is also suggested for other GRDs [4,5,7,8]. Gluten is a ubiquitous protein universally used not only in cereal-based products but also in numerous food and non-food industries [9,10]. Therefore, complete elimination of gluten from the diet is difficult [9,10]. Following a strict GFD also compromises the quality of life (QOL) of CD patients [11].

Gluten protein is made up of primarily two classes of protein i.e., gliadins and glutenins. While gliadin makes the dough viscos, glutenins provide a fine baking quality to wheat [12]. The existence of gliadins and glutenins, as well as the balance of these two forms of proteins, is critical for flour quality. Gliadin is further sub-divided into α-, γ-, and ω-subfractions, out of which α-gliadin primarily contains critical epitopes responsible for CD development [13]. There are two fractions of glutenins i.e., low and high molecular weight glutenins [12]. Gliadin is encoded by multiple gene families that are arrayed at *Gli-2* loci on chromosome 6A, B, and D on specific loci in a repetitive sequence fashion [4,13–16]. α-gliadin contains a 33-mer peptide, particularly rich in proline-glutamine sequences, some of these α-gliadins are responsible for the development of CD. Human intestinal and pancreatic enzymes are unable to completely digest the complex amino acid sequence of α-gliadin which is broken down relatively into larger peptides [4,17]. These peptides pass through intercellular junctions and enter in the lamina propria where tissue transglutaminase enzyme deamidates this gluten fraction. This modified fraction is recognized by HLA-DQ heterodimers attached to antigen presentation cells. The HLA-gluten complex triggers T-cells to induce the pro-inflammatory cascade which eventually leads to CD [17].

Wheat was introduced into the human diet about 10,000–12,000 years ago [18]. 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 *T. monococcum* and rarely consumed by humans nowadays [18]. The tetraploid wheat domesticated simultaneously with diploid wheat, contains two genomes (AA and BB) hence it was termed tetraploid wheat. Durum wheat (*T. durum* or *T. turgidum*) is a tetraploid species of wheat that is used to prepare pasta mostly [18].

Currently used bread wheat/common wheat (*T. 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 *T. tauschii* (DD-genome) [19]. While the introduction of the D-genome improved the bread-making properties, most of the immunogenic peptides in CD are encoded by the D-genome [18]. α-gliadin, encoded on D-genome is more immunogenic and easily recognized by the intestinal T-cells. Preliminary shreds of evidence suggest that primitive wheat (diploid or tetraploid) was safer and less immunogenic compared to currently used hexaploid wheat as ancient wheat varieties had less immune-dominant protein fractions. However, it is strictly dependent on the particular genotype, not on the species [18,20]. Wheat variety with low T-cell stimulatory epitopes may reduce the chances of developing CD. Exposure to an 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 [12,21].

Numerous efforts have been executed to develop a wheat variety with a lower percentage of immunological peptides (α-, ω-, and γ-gliadin), premierly by applying the combination of conventional mutation and breeding methods and RNA interference (RNAi) technology. However, a low-immunogenic wheat variety could not be developed so far. [12,21–24].

In recent years, gene-editing techniques like Zinc Finger Nuclease (ZFN), and Transcription Activator-Like Effector Nucleases (TALEN) have emerged as a promising approach to edit or delete the gluten fractions in wheat [25]. Another promising gene-editing tool i.e., Clustered Regularly Interspaced Short Palindromic Repeats-associated nuclease 9 (CRISPR/Cas9) has evolved as a popular and novel second-generation genome editing

tool in science, medicine, and biotechnology. The CRISPR/Cas9 gene-editing system can remove or reduce the toxic fraction of gluten, resulting in a gluten-free or low-gluten wheat [13]. This gluten-free or low-gluten wheat would be a healthier choice for CD and GRD patients [12]. The use of hypoimmunogenic wheat flour in the preparation of gluten-free food or gluten-free products may also be useful for reducing the increasing burden of gluten cross-contamination [26]. Due to genetic redundancy and genome complexity, wheat biology has straggle behind in adopting CRISPR/Cas9 based genome modifications. The key challenge now is to fully exploit the CRISPR/Cas9 genome editing ability to precisely alter gliadin genes, suppressing their immunogenic capability while maintaining their functionality and organoleptic properties.

So far, only a few studies have reported the application of CRISPR techniques to produce low-immunogenic/gluten-free wheat with novel agronomical traits. To the best of our knowledge, this review is among the first reports to provide an outline of the current status and contribution of CRISPR/Cas9 application in the editing of the wheat genome. This article will help bridge the research gaps that currently exist towards the development of wheat lines devoid of immunogenic gluten.

2. Literature Review

From January to April 2021, published literature related to the application of CRISPR to develop a low-immunogenic wheat variety were searched using the keywords <celiac and CRISPR>, <CRISPR in celiac disease>, <Wheat engineering with CRISPR/Cas9>, <Low-immunogenic wheat and CRISPR>, on the electronic databases like PubMed, Google Scholar, CrossRef, and CiteFactor. We also searched references from the published articles. No publication date was imposed. Only original articles published in the English language applying CRISPR/Cas9 for gene- editing in wheat crops were included. Review articles, protocols, scientific presentations, and Ph.D. thesis were excluded however such articles were used for reading purposes. Following these criteria, in total 68 studies were explored. Of them, 23 articles were found appropriate to the topic.

3. Genome Editing Techniques: Tools That Alter the Genetic Code

Genome editing or gene editing is an advance technique that permits researchers to perform specific alterations in the genome of living cells. During 1970s, the development of genetic engineering (manipulation of DNA or RNA) opened up a innovative possibilities in genome editing.[27].

The main concept behind the genome editing techniques is to employ engineered endonucleases to make site-specific DNA Double-Strand Break (DSB), which is repaired either by non-homologous end joining (NHEJ) or by homologous recombination (HR) [12,25,28,29]. Genome editing techniques have been categoried in two generations (1) first generation (i.e., Mega-nucleases, ZFNs, TALEN) and (2) second-generation (e.g., CRISPR) gene-editing tool [25,30]. CRISPR is the latest gene-editing tool that is highly accurate, rapid, simple, and comparatively cheaper than other gene-editing tools [31,32]. CRISPR/Cas9 system has been successfully applied for plant genome (Arabidopsis, rice, maize, and tomato) improvement and in various human diseases such as gastrointestinal, hematologic, viral, and cancer [13,33]. In a recent study, CRISPR/Cas9 significantly inhibited the tumor cell growth, as well as migration and invasion of breast cancer cells [34].

4. CRISPR/Cas9: A New Era of Genome Editing

The concept of CRISPR/Cas9 was adopted from the defense machinery of bacteria [25,32,35]. When a virus (Bacteriophage) attacks a bacteria, the bacteria capture snippets of the genetic material of the virus and synthesizes DNA segments known as CRISPR arrays [25,32,35]. These CRISPR arrays memorize the virus, on future invasions of the same or similar viruses, the bacteria then synthesizes the RNA segments from the CRISPR arrays that target that virus. Bacteria use the Cas9 enzyme to cleave the targeted viral DNA sequence that eventually neutralizes the virus [36].

CRISPR genome editing system requires designing of 20 nucleotides guide RNA (gRNA) which is complementary to the DNA stretch within the target gene. Along with the gRNA, the system also requires Cas9 endonuclease which together forms a ribonucleoprotein (RNP) complex that creates DSB in complementary DNA sequences [36,37]. In various human diseases, including neurodegenerative conditions, acquired immunodeficiency syndrome, and β-thalassemia, the CRISPR/Cas9 mechanism has been implemented effectively [13,33]. Recently CRISPR/Cas9 has become a promising technique for trait improvement or functional genomics studies in various commercially relevant crops (*Oryza sativa*, *Zea mays*, *Solanum lycopersicum*, *S. tuberosum*, *Hordeum vulgare,* and *T. aestivum*). The use of the CRISPR/Cas9 system in plant genetic engineering is a relatively more contemporary and widely adopted tool for genome editing than ZFNs and TALENs [38,39]. The simplicity, multiplexed mutations, and robustness of CRISPR/Cas9 make it a preferred choice over the first-generation genome editing tools [40].

5. CRISPR/Cas9: The Machinery

CRISPR/Cas9 system presents in diverse living organisms, fundamentally has a comparable core genetic organization [41,42]. They generally have multiple Cas genes encoding Cas protein and several DNA repeat elements interspersed with short "spacer" sequences derived from foreign DNA. The AT-rich spacer sequence constitutes a code for the respective foreign genetic element which is used by the host prokaryotic to quickly identify any homologous sequence subsequently entering the host cell [43].

There are two main components of CRISPR, (1) single guide RNA (sgRNA) which is complementary to the target sequence and (2) the Cas9 gene which is adapted from *Streptococcus pyogenes* (SpCas9) that requires a G-rich (5′-NGG-3′) PAM (protospacer-adjacent motif) site which is responsible for generating DSB at predesigned target DNA site [32,37,44]. 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 [45]. Cas9 protein comprises with two functional domains, (1) large recognition (REC) domain; that is the largest domain and responsible for the gRNA binding, (2) RuvC domain; is a nucleases doamin that cuts the single-standed DNA. 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 [12,36,45]. (Figure 1)

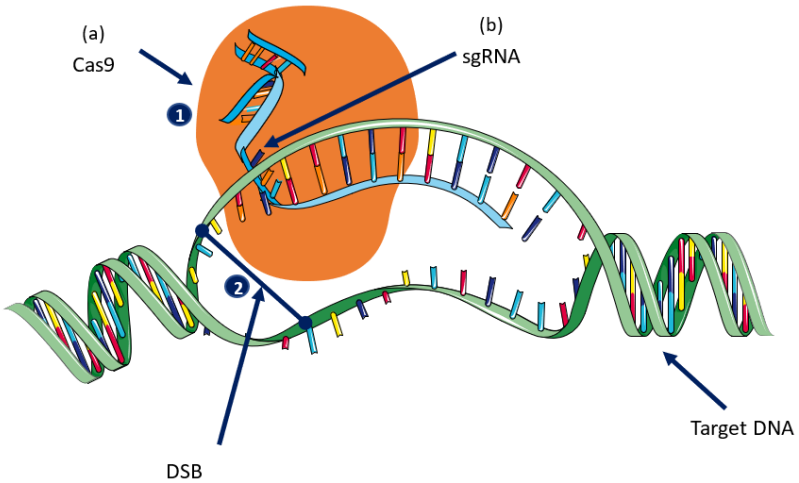

Figure 1. Mechanism of CRISPR/Cas9-mediated gene-editing: there are two key components of CRISPR/Cas9 (**a**) Cas9 and (**b**) single guide RNA (sgRNA) **1:** The Cas9 nuclease is guided to its target DNA by the sgRNA **2:** Cas9 causes a double-strand break (DSB) in the DNA which is repaired using either by a non-homologus joining (NHEJ) or by homologus recombination (HR).

To neutralize foreign DNA in bacterial cells, the CRISPR/Cas9 system works in three stages [46,47].

5.1. Stage I, acquisition stage: The invading DNA is recognized and spacer sequence obtained from the target DNA, the repeated DNA sequence is inserted into the host CRISPR array to build an immunological memory [48,49].

5.2. Stage II, expression stage: The Cas9 protein is expressed at this stage, and the CRISPR array is transcribed into a precursor RNA transcript (pre-crRNA). The pre-crRNA and Cas9 protein are then hybridized by a non-coding trans-activating CRISPR-RNA (crRNA) and processed into a mature RNA unit known as crRNA [50,51].

5.3. Stage III, interference stage: In the final stage, the mature crRNA directs the Cas9 protein to identify the DNA of interest, resulting in the cleavage and degradation of the invading foreign DNA [52,53].

The Cas9 endonuclease cleaves the DNA to generate blunt-ended DSB in the host genome triggering a cellular DNA repair mechanism. The host DNA repair mechanism may either follows a NHEJ with small random insertion/deletion or by HDR thus results in genome editing at the target locus [54]. In NHEJ, a highly error-prone repair mechanism, DSB joins back together with endogenous repair machinery which generally introduces random insertions and deletions of DNA. This could potentially lead to the disruption of the codon-reading frame and often results in gene knockout by forming frameshift and premature stop codon. Alternatively, if donor DNA template homologous to the sequence surrounding the DSB site remains available, the error-free HDR pathway is initiated, whereby precise deletions or insertions of coding sequences can be achieved leading to gene knock-in or deletion. The NHEJ leads to ablation gene mutation and can be used to generate a loss of function effect whereas HDR can introduce precise change in the genome adding specific point mutation or varying length of DNA segment [44,45].

6. CRISPR/Cas9: Challenges and Consequences in the Wheat Genome

CRISPR/Cas9 system is a dominant gene editing tool that has been succesfully applied so far in more than 20 agronomically important crops species to yield improvement, disease resistance, biotic and abiotic stress, etc. [55]. In recent years, the CRISPR/Cas9 system has been employed in model plants like *Arabidopsis thaliana* and *Nicotiana benthamiana*. Subsequently, this genome editing has been employed in major crops like rice, wheat, maize, oilseeds, tomato, soybean, cotton, and potato [56]. Even though the CRISPR/Cas9 method has been validated in various crops, large-scale implementation in editing α-gliadins in wheat is still lacking. One of the major difficulties was the complex wheat genome. Hexaploid wheat *T. aestivum* (Bread Wheat) has a large genome size (approximately 17 Gbp) with a high content of repetitive sequence. This robust sequence prevents the insertion of target mutants in the genome and makes the editing process difficult [12]. Apart from this, the modern wheat is an allohexaploid i.e., it's a result of series of naturally occurring hybridization events among *T. urartu* (A genome donor), *T. speltoides* (B genome donor), and *T. tauschii* (D genome donor) [57,58]. Due to large and complex three homologous copies of genes (A, B, and D) in the genome, targeting such multiple copies of a gene was challenging for gene-editing techniques [12,25].

However, due to the orthologues of the Cas9 gene, CRISPR/cas9 is now capable of targeting multiple genes simultaneously [13,59]. Currently, CRISPR/Cas9 system is being used in the development of a low-immunogenic wheat variety. [12,31,60].

7. Application of CRISPR/Cas9 System in Wheat Genome Editing

In 2014, for the first time, the CRISPR/Cas9 system was used successfully in wheat protoplasts to edit the *TaMLO* gene (Mildew resistance locus O) [61]. The CRISPR TaMLO knockout lines have been successfully established to increase resistance against *Blumeria graminis* f. sp. *Tritici* (Btg) the causal organism of powdery mildew disease. The seventy-two T_0 lines obtained by biolistic particle transformation were analyzed for T7 endonuclease 1 (T7E1) restriction enzyme digestion, four lines reported to be edited for the T7E1 restriction enzyme site [62]. T-DNA based delivery system was commonly used to introduce sequence-specific nucleases (SSNs) and the gRNA. However, DNA-virus-based amplicons were used as an efficient construct delivery method and leading to several-fold

increases in gene targeting efficiencies. The application of Geminivirus-based DNA replicons, such as a wheat dwarf virus (WDV) in wheat, resulted in a 12-fold increase in CRISPR/Cas9 expression compared to the ubiquitin reference gene, suggesting that it could be a future tool for genome engineering for complex genomes [63]. In another study, Kim et al., (2018) demonstrated gene-editing in wheat protoplasts for *dehydration-responsive element-binding protein 2* (*TaDREB2*) and *ethylene-responsive factor 3* (*TaERF3*) using wheat U6 snRNA promoter [60]. They successfully transfected nearly 70% of protoplasts and confirmed the expressions of these edited genes with the T7 endonuclease assay. The two major pitfalls of CRISPR-mediated gene-editing in crops (CMGE) were transgene integration and off targeting into genome. Off-target mutations were more common in crops with higher ploidy levels, as well as genes with a large number of paralogs. This shortcoming was overcome by using biolistic delivery method of CRISPR/Cas9 ribonucleoproteins (RNPs). However, the RNP-based biolistic delivery offers a transient expression of CRISPR/cas9 and, it also *reduces the* chance of *off-target* effects [31]. Later in 2017, Liang et al., demonstrated the use of CRISPR/Cas9 RNP complex genome editing of grain morphometric traits like grain length (GL), width (GW) genes *TaGW2,* and *TaGASR7* in *T. aestivum*. This complex reduced off-target effects as no off-targets were detected in the mutant *T. aestivum* population and in addition, the complex gets degraded in vivo. This DNA-free editing method had an advantage over traditional backcross breeding which is a laborious and time-consuming procedure [64]. However, this method had some limitations including low-efficiency rates compared to CRISPR/Cas9 DNA binary delivery systems. RNP method is a more economical approach to achieve CRISPR/Cas9 based genome editing in perennial crop species if these limitations are overcome. Similarly, Wang W. et al., (2018) demonstrated multiplexed gene-editing of three wheat genes, *TaGW2* (a negative regulator of grain traits), *TaLpx-1* (lipoxygenase, which confers resistance to *Fusarium graminearum*), and *TaMLO* (loss of function, confers resistance to powdery mildew resistance) using wheat U3 snRNA promoter [59]. Genome editing efficiency was validated in wheat protoplasts and the DNA was evaluated for mutations by next-generation sequencing (NGS) followed by *Agrobacterium*-mediated transformation and mutant screening. T_0, T_1, T_2, and T_3 were then subjected to statistical and phenotypic analysis and three homeologous copies in wheat were observed for gene-editing efficiencies. In another study, the male sterility gene i.e., *Ms1* (male sterility 1) was targeted by CRISPR/Cas9 vectors resulting in the generation of complete sterility in commercial wheat cv. Fielder and Gladius [65]. Sánchez-León et al., 2018 used particle bombardment to demonstrate the potential of CRISPR/Cas9, this time with two gRNAs delivered separately. They focused on genes that encode α-gliadins, seed storage proteins that have an epitope linked to CD. Twenty-one mutant lines in bread wheat and six in durum wheat were developed, both of which showed a significant reduction in α-gliadins and had up to 35 genes edited in a single line [12]. Howells et al., (2018), delivered gRNAs into wheat cells using *Agrobacterium tumefaciens*-mediated transformation, for example, to target the *TaPDS* gene, which encodes phytoene desaturase [66]. Interestingly, Zhang et al., (2019) generated heritable targeted mutation in *TaPinb*, *TaDA1*, *TaDA2*, and *TaNCED1*. The combination of the *Agrobacterium*-mediated transformation process and the CRISPR/Cas9 gene-editing system greatly increased the mutagenesis efficiency in T_0 generation. High editing frequency was observed in the subsequent T_1 and T_2 generations. Since CRISPR/Cas9 activity is stable through generations, *Agrobacterium*-mediated transformation in wheat proved an ideal approach for genome editing [67]. Furthermore, *Agrobacterium*-mediated transformants contain only one or a few copies of the transgene, transgene-free mutant lines are reasonably simple to acquire [68]. Kamiya et al., (2020) developed PCR-RFLP, a rapid method for detecting edited mutations in wheat that was validated by genomic clone sequencing. Three *TaNP1* homoeo-alleles, which encode a putative glucose-methanol-choline oxidoreductase and are needed for male sterility, were edited using the optimized CRISPR/Cas9 method. It was also demonstrated that having only one wild-type copy of each of the three *TaNP1* genes was enough to keep male fertility [69]. In a latest study, in order to reduce the expression of asparagine synthetase in the grain without affecting its expression in any other

part of the plant, Raffan et al., (2021) targeted the *TaASN2* gene in *T. aestivum* cv. Cadenza using CRISPR/Cas9 system. The study provided strong evidence that very low asparagine commercial wheat varieties could be produced, allowing for the development of lower-acrylamide bread, cereals, biscuits, and other wheat-based foods [70].

Above mentioned studies successfully demonstrates that CRISPR/Cas9 system has emerged as an effective tool to enables precise genome manipulation for the development of new wheat cultivars with improved novel traits. These studies documented that CRISPR/Cas9 has been successfully employed in the wheat genome to improve disease resistance, stress tolerance, increase yield, and nutritional improvement. We have summarized the twenty-three studies **that used** CRISPR/Cas9 mediated gene-editing in wheat varieties in Table 1.

Table 1. Summary of functionally validated CRISPR/Cas9 based genome editing in wheat varieties**.**

S. No	Cultivar or Genotype	Target Gene (s)	Gene Function	Delivery Mode	SgRNA Promoter Used	Reference
1	*T. aestivum* cv. Cadenza	*TaASN2*	Genes encode for asparagine synthetases enzyme required in asparagine synthesis	Biolistic transformation	*Ubi-1*	Raffan et al., (2021) [70]
2	*T. aestivum* line H29 cv. Fielder & Ningchun4	*TaWaxy* & *TaMTL*	Pollen-specific phospholipase	*Agrobactrium tumefaciens* mediated transformation	*OsU6a*, *TaU3*, & *TaU6*	Liu et al., (2020) [71]
3	Wheat variety CB037	*TaNP-A1, TaNP-B, TaNP-D1*	Express in the tapetum & required for male fertility	Biolistic & protoplast mediated transformation	*TaU6* & *TaU3*	Li et al., (2020) [72]
4	Common wheat (*T. aestivum* L.)	*TaQsd1, TraesCS4A02G110300 (IWGSC 2018)*	Control seed dormancy in wheat	Biolistic transient expression & *A. tumefaciens* mediated transformation	*TaU6*	Kamiya et al., (2020) [73]
		TaLOX2	Encodes for lipoxygenase 2; Grain development & growth			
5	*T. aestivum* cv. Fielder	*TaABCC6* & *TaNFXL1*	Susceptibility to Fusarium head blight (FHB)	Protoplast transformation	*TaU6*	Cui et al., (2019) [74]
		TansLTP9.4	FHB resistance			
6	*T. aestivum* cv. Fielder	*EPSPS*	The key enzyme involved in the metabolism of aromatic amino acid through the shikimate pathway	Protoplast transformation	*TaU6*	Arndell et al., (2019) [75]
7	*T. aestivum* cv. Fielder	*TaPinb*	Control grain hardness	*A. tumefaciens* (EHA105) mediated transformation	*TaU3*	Zhang et al., (2019) [67]
		TaDA1, TaDA2	Negative regulates seed & organ size			
		TaNCED1	Key enzyme in ABA biosynthesis pathway which confers resistance to drought stress			
8	*T. aestivum* cv. Fielder	*TaQsd1*	Control seed dormancy in wheat	*A. tumefaciens* (EHA101) mediated transformation	*OsU6*	Abe et al., (2019) [68]
9	*T. aestivum* cv. Kenong199 or Kenong9204	*TaALS, TaACCase*	The absence of the gene provides herbicide tolerance	Biolistic transformation	*TaU6*	Zhang et al., (2019) [76]
10	*T. aestivum* cv. Fielder & cv. Gladius	*TaMs1*	Encodes a GPI, which is required for pollen exine development	*A. tumefaciens* mediated transformation	*TaU6*	Okada et al., (2019) [65]
11	*T. aestivum* cv. Fielder	*TaCKX2-1, TaGLW7, TaGW2, TaGW8*	Wheat grain-regulatory genes	*A. tumefaciens* mediated transformation	*TaU6*	Zhang et al., (2019) [77]
12	*T. aestivum* cv. Fielder	*TaPin a* & *b*	Control grain hardness & contributes to anti-fungal properties	*A. tumefaciens* mediated transformation	*TaU6* & *TaU3*	Zhang et al., (2018) [44]
		TaWAXY or GBSS	Key enzyme in amylase biosynthesis			
		TaDA1	Negatively regulates seed & organ size by restricting the period of cell proliferation			
13	*T. aestivum* cv. Bobwhite	*TaGW2*	Negative regulator of grain weight, grain size enlargement, especially increased kernel width	Protoplast transformation	*TaU6*	Wang et al., (2018) [59]
		TaLpx-1	Encodes 9-lipoxygenase, silencing results in resistance to *Fusarium graminearum*			
		TaMLO	Knockout mutants provide resistance to powdery mildew			

14	*T. aestivum* cv. Chinese Spring	*TaPDS*	Reduction or loss of function results in a photobleaching phenotype	*A. tumefaciens* mediated transformation	*TaU6*	Howells et al., (2018) [66]
15	*T. aestivum* cv. Fielder or SBC0456D	*TaMs45*	Contribute to male fertility	*A. tumefaciens* mediated transformation	*TaU6*	Singh et al., (2018) [78]
16	Bread wheat, BW208 & THA53, & Durum wheat cv. Don Pedro	α-gliadin	Storage protein, adds to dough viscosity/plasticity & contains immunogenic epitopes for CD	Biolistic transformation	*TaU6*	Sánchez-León et al. (2018) [12]
17	*T. aestivum* cv. Chinese spring	*TaDREB2*	TF induced under water-deficient condition	Protoplast transformation	*TaU6*	Kim et al., (2018) [60]
		TaERF3	TF promotes tolerance under salt and drought stress			
18	*T. aestivum* cv. Bobwhite & AC Nanda	*TaLox2*	Encodes for lipoxygenase enzyme which hydrolyzes linoleic acid, α-linolenic acid & arachidonic acid	Neon transfection of protoplasts & microspores	*TaU6*	Bhowmik et al., (2018) [79]
19	*T. aestivum* cv. Bobwhite	*TaUbi, TaMLO*	Major responsible for powdery mildew vulnerability	WDV & Biolistic transformation	*TaU6*	Gil-Humanes et al., (2017) [63]
20	*T. aestivum* cv. Kenong 199	*TaGW2*-A1, -B1 & -D1	Negatively regulates grain weight and width	Biolistic transformation	*TaU6*	Liang et al., (2017) [31]
21	*T. aestivum* cv. Bobwhite & cv. Kenong199	*TaGASR7*	Gene controls the expression of grain length, with pleiotropic effects on grain weight & yield	Biolistic transformation	*TaU6*	Zhang et al., (2016) [80]
		TaDEP1	Gene expression controls panicle size			
		TaLOX2	Encodes for lipoxygenase 2 & plays a critical role in grain storage and seed vigor			
		TaNAC2	TF promotes multiple abiotic stresses tolerance			
		TaPIN	Encodes for puroindoline gene & plays an important role in controlling the grain hardness			
		TaGW2	Negative regulator of grain weight, grain size enlargement, especially increased kernel width			
22	*T. aestivum* L.	*TaMLO-A1, TaMLO-B1* & *TaMLO-D1*	Loss of function confers resistance to Powdery mildew	Biolistic transformation	*TaU6*	Wang et al., (2014) [62]
23	*T. aestivum*	*TaINOX*	Biogenesis of plant cell wall	*A. tumefaciens* (GV3101) mediated transformation	TaU6 and CaMV35s	Upadhyay et al., (2013) [81]
		TaPDS	Involved in carotenoid biosynthesis that protects chlorophyll from photobleaching			

Abbreviations: ABA, Abscisic acid; TF, Transcription Factor; CD, Celiac Disease; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GPI, glycosylphosphatidylinositol; *OsU*, *O. sativa* small nucleolar RNA (snoRNA) promoters; *TaU*, *T. aestivum* snoRNA promoters; *Ubi-1*, *Z. mays* ubiquitin promoter.

8. RNA Interference (RNAi): Biology

The discovery of RNA-induced gene silencing provided a feasible alternate gene analysis technique by simultaneously knockdown the expression of multiple related gene copies. RNAi or RNA-silencing was discovered in *Caenorhabditis elegans* and plants during the late '90s as a post-transcriptional gene silencing (PTGS) mechanism that targets specific messenger RNA (mRNA) sequences and downregulates protein expression [29,82–84]. RNA interference involves four main stages (1) double-stranded RNA cleavage by the dicer, (2) silencing complex (RISC) development, (3) silencing complex activation, and (4) mRNA degradation.

The first step in RNAi is the transmission of dsRNA into the cell, which is completely homologous to the target gene in sequence. The Dicer enzyme recognizes dsRNA and converts it into double-stranded short interfering RNA (siRNA) nucleotides of varying lengths in an ATP-dependent reaction, depending on the species. In the second step, the siRNAs produced by Dicer are integrated into the RNA-induced silencing complex (RISC), a multicomponent nuclease complex that is inactive in this form to conduct RNAi [29,85]. In an ATP-dependent process, a helicase unwinds the siRNA duplex and further remodeling the complex to form an effective RISC in the third step. The final step is to recognize and cleave mRNA that is complementary to the siRNA strand present in RISC.

The target mRNA is cleaved into 22 nucleotide-long fragments, resulting in gene suppression or alteration of the gene expression [86]. When the cleavage comes to an end, the RISC leaves and the siRNA is ready to be used in another period of mRNA recognition and cleavage [87,88].

9. Role of RNAi in Modifying the Wheat Genome

Wheat RNAi has been successfully used to target a wide range of genes to date, but it was also used to down-regulate protein encoded by multigene families, such as gliadins and glutenins [89,90]. In a short communication published by Gil-Humanes et al., 2008 the authors used RNA interference to suppress the expression of particular γ-gliadins, demonstrating the feasibility of systematically silencing specific groups of gluten proteins. There were seven transgenic lines, all of which displayed decreased γ-gliadin content. The seven transgenic plants were fully fertile and the grain morphology and seed weight were comparable to the wild type. The proportion of γ-gliadins was decreased by about 55–80% in the BW208 lines and by about 33–43% in the BW2003 lines as a result of this silencing [85]. In another influential study published in 2010, Gil-Humanes et al., have down-regulated the gliadin expression (up to 63–93% for α-gliadin and 35–81% for ω-gliadin) in bread wheat by designing a set of hpRNA containing a fragment of 361 bp widely conserved among α-, ω-, and γ- gliadins. There was a 1.5–2 log reduction in the sum of DQ2-α-II and DQ2-γ-VII epitopes and at least 1 log reduction in the amount of DQ8-α-I and DQ8-γ-I epitopes in five of the transgenic lines. For three of the transgenic wheat lines, the whole gluten extracts were unable to produce T-cell responses and for six transgenic lines had decreased responses [91]. Gil-Humanes et al., again in 2014 used flour from these transgenic wheat lines to develop a high-quality bread. The baking and sensory properties, as well as overall approval, of the reduced-gliadin breads were comparable to those of regular flour, but with up to 97% less gliadin content. Furthermore, low gliadin flour enhanced nutritional properties because their lysine level was considerably higher than that of regular wheat [92].

In a recent study, Haro et al., (2018) have compared the digestibility of low-gliadin wheat (E82, low gliadin content, and reduced LMW glutenins) developed by the RNAi system from regular gluten-free bread in a subset of patients with no-celiac gluten sensitivity (NCGS). The findings indicated that eating low-gliadin E82 bread for one week was well acceptable by NCGS patients, as clinical effects were similar to those seen with gluten-free bread, and no variations in sensory parameters were observed. The data showed that the consumption of E82 bread, does not cause adverse clinical symptoms, induces positive changes to the composition of the gut, increases butyrate-producing bacteria, and promotes the bacterial profile of the intestines which plays a major role in the improvement of the gut permeability in NCGS patients. However, this study did not address the relationship between the bacterial and fungal species of the gut microbiota. Further studies are needed to investigate the bacterial and fungal microbiota modification in the gut on the consumption of E82 bread [93].

These study findings indicate that RNAi is effective in reducing the levels of gliadins in wheat which would be safer for gluten-intolerant consumers. However, it is still debatable if these wheat lines will become commercially viable or whether the discoveries can be converted into something of economic utility.

10. Applications of CRISPR/Cas9 and RNAi: A Comparative Analysis

Gene modifications are powerful tools that have been widely used over the past decades to understand fundamental biological processes of interest and their function. RNAi has previously been the major dominating genetic tool for manipulating genes and performing genetic function studies in various areas of crop development. However, the rapid growth and use of CRISPR/Cas9 has been successfully applied in many agronomic crops. Both RNAi and CRISPR/Cas9 are useful tools for modifying genomic DNA and changing genetic information, including gain-of-function and loss-of-function. CRISPR/Cas9 and RNAi are widely explored from a technical and methodological

standpoint (Figure 2). A comparison of the scope of CRISPR/Cas9 and RNAi in research and practical studies has been discussed below.

10.1. Knockout vs. Knockdown: CRISPR causes gene knockouts, which occur when DSB is made within the coding region of the gene[94]. This DSB triggers NHEJ or HDR [95]. RNAi reduces or knocks down gene expression at the post-transcriptional level by targeting RNA, where it generates hypomorphic phenotype in contrast to the true null knockout possible with CRISPR/Cas9.

10.2. Ease of Design: The designing of a siRNA requires the sequence information of the corresponding mRNA transcript. siRNA is designed to target any transcript at almost any locus but its activity is influenced by other factors like the structure of the mRNA target region, base preferences, and overall siRNA G/C content. The design of a siRNA is a critical component of an effective RNAi experiment. CRISPR, on the other hand, requires information about the genomic DNA sequence. CRISPR system such as CRISPR/Cas9 requires the protospacer adjacent motif (or PAM), a short DNA sequence required to cleave the targeted DNA. Depending on the type of Cas9, the PAM sequence recognizes the 5′-NGG-3′ site (where "N" can be any nucleotide base) [96].

10.3. Timespan: The mode of action differs between CRISPR/Cas9 and RNAi, which greatly impacts the duration of gene expression. siRNA knockdown exhibits significant gene repression in only 24 h of the treatment. However, genome editing with CRISPR/Cas9 may result in a permanent effect, which usually requires the selection of cells with desired InDels (insertion-deletion mutation) in all alleles, a time-consuming process depending on the specific need [97].

10.4. Flexibility: Targeted gene-editing, especially CRISPR/Cas9 is heritable, i.e., once it introduces the change in the genome of the host cells; its physiological effect is passed on to the next generation. RNAi, unlike CRISPR/Cas9, does not result in a stable gene fragment, mutation, or inactive gene [98]. The in vivo application of RNAi is limited to the instances where gene expression is suppressed post-transcriptionally.

10.5. Off targets: Since the discovery of RNAi, off-targets is one of the biggest limitations. siRNA induced silencing of non-target mRNA with a limited sequence complementary, via interaction with 3′UTR. However, it has been discovered that a single siRNA could potentially repress hundreds of transcripts with limited complimentary. But the CRISPR/Cas9 system also had some sequence-specific target effects which were overcome during the short time spam. This shortcoming was rectified by the use of Cas9-nickase, a mutation in one of the Cas9 nucleases, reduces off targeting by 50-1500 folds [99]. While optimal siRNA design and chemical modifications have reduced RNAi's off-target activity, a recent comparative study found that CRISPR/Cas9 is less susceptible to off-target effects than RNAi [100].

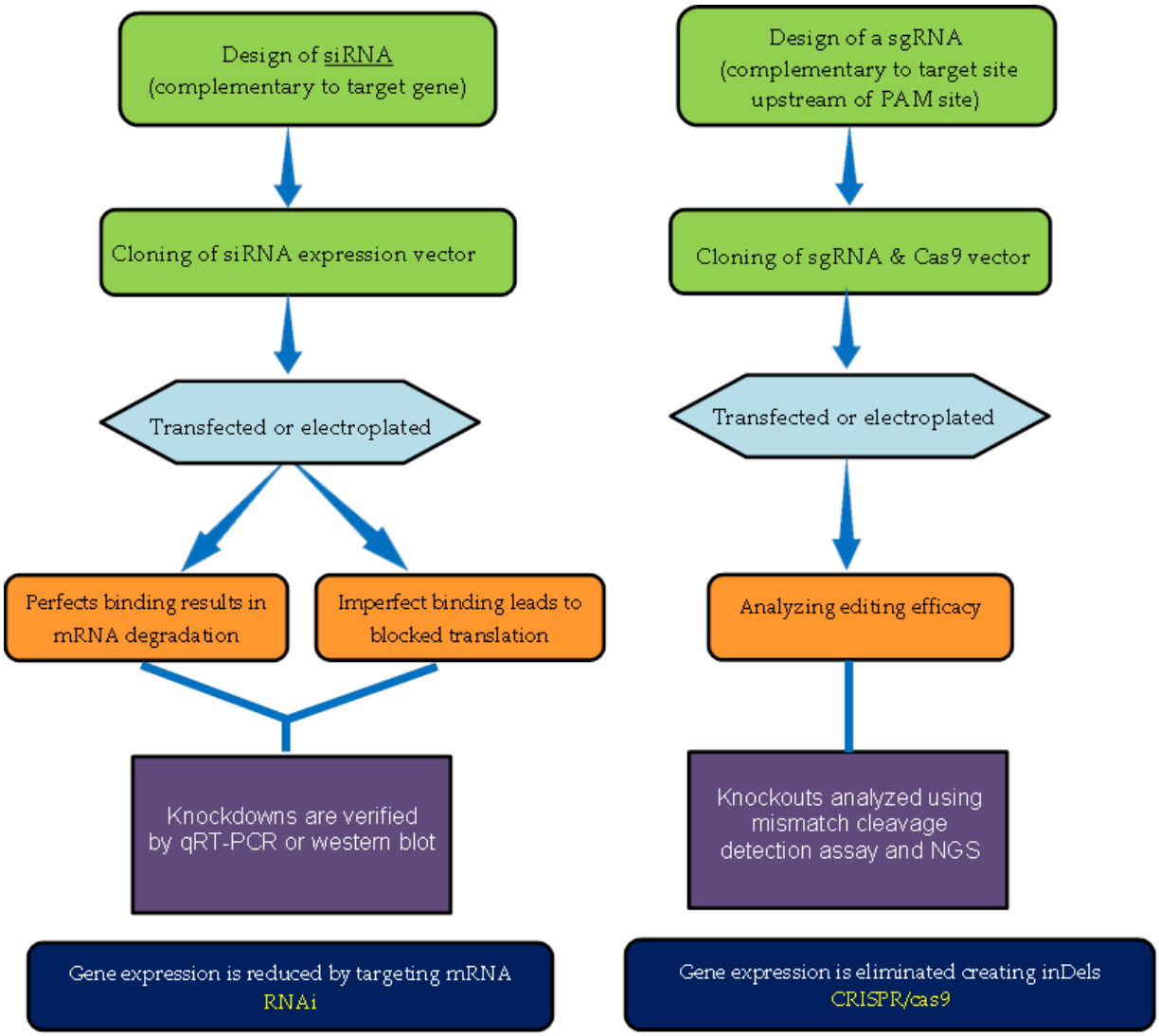

Figure 2. Schematic representation of RNAi and CRISPR/Cas9 experimental workflow.

Abbreviations: NGS, next-generation sequencing; inDel, insertion-deletion mutation.

11. CRISPR/Cas9 Is A Method-of-Choice for Wheat Genome Editing

The recent emergence of multiple technologies for modifying gene structure has reformed agriculture and improved crops that were not possible with conventional breeding procedures alone. These genetically modified crops brought huge economic and environmental benefits and are widely accepted across the world. Over the past decade, the RNAi technique has been widely used in both dicotyledon and monocotyledon to improve plant growth and productivity, impart resistance against pathogens, and tolerance against various biotic stresses. The RNAi or Post-Transcriptional Gene Silencing (PTGS) is a cellular mechanism conserved in most eukaryotic organisms, which leads to the loss of functionality of a gene by blocking the messenger RNA (mRNA) molecules needed for protein formation.

Since RNA expression constructs are typically delivered as transgenes, through plant transformation, or as part of virus vectors, they must go through genetically modified organism (GMO) regulatory procedures to gain commercial approval. Several other techniques for stable genetic modifications, collectively known as gene-editing techniques, have been developed in parallel to the production of RNAi [101]. CRISPR is one such novel second-generation genome editing system that has been exploited to generate desired mutations, facilitating the development of crops with any given desirable trait. In the last decade, due to its simplicity, speed and efficiency, CRISPR/Cas9 quickly became

a standard technique for modifying endogenous genes in almost all crop species. The CRISPR/Cas9 system has target specificity as the target sites are recognized by the Watson and Crick model and off-target sites are identified through sequence analysis [102]. CRISPR/Cas9 represents significant technical advances for genetic engineering, but attempts must be taken to increase their productivity in a variety of plant species with large, complex genomes.

While the utility of the CRISPR/Cas9 has been studied in many diploid plants, its applicability in polyploidy crops with complex genomes (wheat) is still a challenge. Wheat is an allohexaploid consist of three sets of closely associated homogeneous genomes [37,59]. Therefore, simultaneously targeting three or even more copies of a gene is a problem for editing wheat genomes, and attempting to knock out any of a gene's copies does not result in phenotypic modifications due to genome buffering. Wheat, on the other hand with a large genome and high content of repetitive DNA (80–90%), makes it unusually recalcitrant to introduce targeted mutations. However, due to the availability of new orthologs of the Cas9 gene, sgRNA design in the CRISPR/Cas9 system can be effectively programmed to target several genes.

Another concern is that there are only a few wheat varieties that can be easily transformed, which restricts the use of CRISPR in wheat. However, there are well-established protocols for transformation of the CRISPR/Cas9 construct using *Agrobacterium*-mediated and bombardment or biolistics delivery method [35]. In addition, using recently designed CRISPR-based multiplex genome editing toolkits it is possible to accomplish simultaneous multiplex targeted modifications by co-transforming multiple sgRNAs. Evidence from published data showed that CRISPR/Cas9 technique has been successfully applied to numerous wheat varieties to engineer novel agronomic traits associated with yield, quality, and resistance to biotic and abiotic stresses, etc. CRISPR/Cas9 is highly desirable for achieving the goal of editing α-gliadin genes in the development of wheat lines with fewer gluten genes and/or gluten genes with inactivated CD epitopes in bread wheat [13].

12. Discussion

Celiac disease is a complex disorder in which the function of a major non-genetic factor, gluten, has been well established. A life-long GFD is a sole cure for CD [7]. However a GFD, on the other hand, is difficult to follow because gluten is a commonly used food additive that can be found in items that did not initially contain gluten [10,103]. Furthermore, gluten-free products can be less healthy nutritionally since they are made with high levels of fat and sugar to create a texture that resembles the normal and unusual viscoelastic properties of wheat. Additionally, studies have linked GFD to lower consumption of dietary fiber and some commercially available GFPs have lower vitamin B, folate, and iron content[104]. Moreover, the exclusion of gluten from the diet of CD patients reduces the QOL [11]. Rigorous efforts have been carried out to explore an alternative treatment that allows consumption of wheat to CD patients [17]. The use of a special wheat variety devoid of T-cell stimulatory epitopes may be a viable and successful alternative option. Currently, the only safe alternative could be the development of a "low-gluten/gluten-free" wheat variety that does not contain toxic peptides while retaining the basic properties of wheat [17,105,106].

Since bread wheat has a complicated hexaploid genome, successful breeding of this crop is heavily reliant on the understanding of functional genomics. Advanced crop functional genomics, which can show how wheat genetic determinants function, must now be complemented with existing modern breeding efforts. Plant biologists can alter the structures and functions of selected key genes through "genetic manipulation" based on their understanding of functional genomics. RNAi and CRISPR/Cas9 are two advanced technologies that can be used to modify or remove CD epitopes from wheat gluten. RNA silencing technique showed favorable results in this regard. Several research groups explored the possibilities of using RNAi in silencing the toxic fragments of gliadin and found promising results [85,91,107]. In a fundamental study, Gil-Humanes et al. used RNA interference to reduce gliadin gene expression by 97%, therefore, preventing the stimulation

of T cells from CD patients without compromising seed germination or dough quality [91]. The RNAi wheat line (E82) developed by Javier Gil-Humanes and colleagues, was exceptional for its low ability to produce an immunogenic response and retaining its organoleptic and agricultural properties. The study was conducted in the volunteer of NCGS patients compared with GFD to test the acceptability, digestibility, and safety of bread made from the wheat flour of the E82 line with all gliadins strongly downregulated. Furthermore, in non-celiac wheat sensitivity patients, eating bread made with this low-gliadin line encourages a stronger gut microbiota profile than gluten-free bread [92].

Since the transgenic RNAi construct persists in the wheat genome to silence the genes, these plants are subjected to GM control, which is costly, time-consuming, and unpredictable in the European Union (EU) [21,26,108]. Unlike other breeding methods, the implementation of genetic transformation is strongly regulated in the EU. This contradicts the fact that the cultivation of GMOs is essentially prohibited in the EU, but importation is permitted [109]. As a result of this stringent regulation, the general populations are concerned about GMOs on a variety of levels, including their environmental impact and whether GM foods pose any health risks.

Emerging targeted genome editing technologies offer plant breeders a new and effective tool. For genome editing, SSNs were used to alter the target position of genes present in the genome. SSN, like CRISPR/Cas9, causes DSB, which are repaired using an NHEJ or HR [54,110]. Unlike transgenic modifications, which require the insertion of foreign DNA sequences into a genome, gene-editing may produce genetic variation through precise and direct changes in genes of interest without integrating foreign DNAs or, if so, null segregants containing no recombinant DNA but maintain the desired mutations can be easily retrieved. Instead of being categorized as GMOs, such edited plants could be considered non-transgenic plants. Moreover, It is expected that the Court of Justice of the European Union (ECJ) will exempt the CRISPR/cas9 modified crops from existing European law that has limited the planting and sale of GM crops.[111]

In plants, CRISPR/Cas9 has already been shown to be a very high-efficient genome editing system [112–114]. The hexaploid genome and large genome size are the major obstacles to CRISPR use in wheat biology. However, because of CRISPR/Cas9, high efficiency, it is possible to acquire mutations in multiple genomes in a single polyploid plant. Finally, multiplexed genome editing using the CRISPR/Cas9 library can be easily accomplished using the monomeric Cas9 protein and a variety of sequence-specific gRNAs [25,59]. Moreover, genome editing through CRISPR/Cas9 entails a few simple steps that enable smaller laboratories with basic plant transformation to perform genome editing in crop plants. The ease of use of CRISPR/Cas9 programming and its potential for multiplexed target identification have fueled the success of this low-cost and easy-to-use technology. According to some research, while CRISPR/Cas9 can cleave a target site, it can also cleave sites with a few mismatches to the target site [61]. In gene therapy, this off-target effect is a major problem, but it may not be a concern in plant biotechnology. Backcrossing or crossing with wild-type plants could be used to remove the putative off-target mutation. Furthermore, it is advisable to use web-based software to develop target sites in order to mitigate off-target mutations by exploiting computation.

Susana Sánchez-León et al., utilized CRISPR/Cas9 genome editing to technology to reduce the number of α-gliadins in the seed kernel precisely and effectively, resulting in gluten-free bread and durum wheat lines [12]. Interestingly, the bread wheat line (plant 10) had the highest decline in α-gliadins (82%) and γ-gliadins (92%) as well as the highest overall gliadin reduction (82%). Amongst the durum wheat lines, (plant 2) had the highest overall gliadin reduction (69%). 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 palate of GFD, etc. [8,115]. Safe wheat variety for CD patients will be helpful to eradicate this problem and CRISPR/Cas9 technology has the potential to produce such a variety of wheat [26]. However, CRISPR

modified wheat flour may lead to problems to dough formation, that need to be resolved. Nonetheless, multiple studies) support the fact that CRISPR/Cas9 gene-editing has overcome the current wheat genome complexity for genetic improvement **(**Table 1). The use of CRISPR/Cas9 for gene knockout and the Cas9 system for expression regulation of any gene of interest would aid in the development of non-transgenic wheat plants. CRISPR technology is evolving, and existing systems are being engineered to include innovative capabilities; exciting new CRISPR systems with novel functions are also being discovered.

13. Conclusions

CRISPR/Cas9 system is capable of editing complex hexaploid wheat genome (*T. aestivum)*. Availability of whole-genome sequence information of wheat along with the advancement in CRISPR/Cas9 technique could provide possibilities to develop a "hypo-immunogenic-wheat variety". CRISPR/Ca9 could be a breakthrough for providing a promising dietary treatment for celiac disease. However, until now, only a limited number of studies have applied the CRISPR/Cas9 system to develop low-gluten wheat. Further studies are required to apply the CRISPR/Cas9 gene-editing system efficiently for the development of celiac-safe wheat variety and to establish it as a "tool to celiac safe wheat.".

Supplementary Materials: The manuscript contains all relevant data.

Author Contributions: Conceptualization, A.K.V., S.M., A.T. and C.C.; software (CRISPR/Cas9 representative figure), A.K.V. and A.T.; Data Curation, A.K.V., S.M., A.T., A.S., E.L., C.M., G.N.C., and S.G.; Writing—Original Draft Preparation, A.K.V., S.M. and A.T.; Writing—Review & Editing, A.K.V., S.M., A.T., A.S., E.L., C.M., G.N.C., and S.G.; final approval of the version to be published, E.L. and C.C.; an overall guarantor of the manuscript, C.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement:

Data Availability Statement:

Conflicts of Interest: Carlo Catassi is a scientific consultant to Dr. Schär and Takeda, all other authors declare no conflict of interest.

References

1. Shewry, P.R. Wheat. *J. Exp. Bot.* **2009**, *60*, 1537–1553, doi:10.1093/jxb/erp058.
2. Https://Www.Statista.Com/Topics/1668/Wheat/ Accessed on 27 June 2020.
3. Gatti, S.; Lionetti, E.; Balanzoni, L.; Verma, A.K.; Galeazzi, T.; Gesuita, R.; Scattolo, N.; Cinquetti, M.; Fasano, A.; Catassi, C.; et al. Increased Prevalence of Celiac Disease in School-Age Children in Italy. *Clin. Gastroenterol. Hepatol.* **2019**, doi:10.1016/j.cgh.2019.06.013.
4. Sapone, A.; Bai, J.C.; Ciacci, C.; Dolinsek, J.; Green, P.H.R.; Hadjivassiliou, M.; Kaukinen, K.; Rostami, K.; Sanders, D.S.; Schumann, M.; et al. Spectrum of Gluten-Related Disorders: Consensus on New Nomenclature and Classification. *BMC Med* **2012**, *10*, 13, doi:10.1186/1741-7015-10-13.
5. Catassi, C.; Elli, L.; Bonaz, B.; Bouma, G.; Carroccio, A.; Castillejo, G.; Cellier, C.; Cristofori, F.; de Magistris, L.; Dolinsek, J.; et al. Diagnosis of Non-Celiac Gluten Sensitivity (NCGS): The Salerno Experts' Criteria. *Nutrients* **2015**, *7*, 4966–4977, doi:10.3390/nu7064966.
6. Verma, A.K.; Singh, A.; Gatti, S.; Lionetti, E.; Galeazzi, T.; Monachesi, C.; Franceschini, E.; Ahuja, V.; Catassi, C.; Makharia, G.K. Validation of a Novel Single-Drop Rapid Human Leukocyte Antigen-DQ2/-DQ8 Typing Method to Identify Subjects Susceptible to Celiac Disease. *JGH Open* **2018**, *2*, 311–316, doi:10.1002/jgh3.12090.
7. Ciacci, C.; Ciclitira, P.; Hadjivassiliou, M.; Kaukinen, K.; Ludvigsson, J.F.; McGough, N.; Sanders, D.S.; Woodward, J.; Leonard, J.N.; Swift, G.L. The Gluten-Free Diet and Its Current Application in Coeliac Disease and Dermatitis Herpetiformis. *United European Gastroenterol J* **2015**, *3*, 121–135, doi:10.1177/2050640614559263.
8. Lionetti, E.; Catassi, C. New Clues in Celiac Disease Epidemiology, Pathogenesis, Clinical Manifestations, and Treatment. *Int. Rev. Immunol.* **2011**, *30*, 219–231, doi:10.3109/08830185.2011.602443.

9. Sharma, G.M.; Rallabhandi, P.; Williams, K.M.; Herrmann, M.; Sadrieh, N. Gluten Quantitation in Cosmetic Products by Enzyme-Linked Immunosorbent Assay Available online: http://www.ingentaconnect.com/content/aoac/jaoac/2016/00000099/00000003/art00002;jsessionid=14qariq4i3ll9.x-ic-live-01 (accessed on 16 September 2018).
10. Verma, A.K.; Lionetti, E.; Gatti, S.; Franceschini, E.; Catassi, G.N.; Catassi, C. Contribution of Oral Hygiene and Cosmetics on Contamination of Gluten-Free Diet: Do Celiac Customers Need to Worry About? *J. Pediatr. Gastroenterol. Nutr.* **2019**, *68*, 26–29, doi:10.1097/MPG.0000000000002129.
11. Wolf, R.L.; Lebwohl, B.; Lee, A.R.; Zybert, P.; Reilly, N.R.; Cadenhead, J.; Amengual, C.; Green, P.H.R. Hypervigilance to a Gluten-Free Diet and Decreased Quality of Life in Teenagers and Adults with Celiac Disease. *Dig Dis Sci* **2018**, *63*, 1438–1448, doi:10.1007/s10620-018-4936-4.
12. Sánchez-León, S.; Gil-Humanes, J.; Ozuna, C.V.; Giménez, M.J.; Sousa, C.; Voytas, D.F.; Barro, F. Low-Gluten, Nontransgenic Wheat Engineered with CRISPR/Cas9. *Plant Biotechnol J* **2018**, *16*, 902–910, doi:10.1111/pbi.12837.
13. Jouanin, A.; Schaart, J.G.; Boyd, L.A.; Cockram, J.; Leigh, F.J.; Bates, R.; Wallington, E.J.; Visser, R.G.F.; Smulders, M.J.M. Outlook for Coeliac Disease Patients: Towards Bread Wheat with Hypoimmunogenic Gluten by Gene Editing of α- and γ-Gliadin Gene Families. *BMC Plant Biol* **2019**, *19*, 333, doi:10.1186/s12870-019-1889-5.
14. Sollid, L.M.; Qiao, S.-W.; Anderson, R.P.; Gianfrani, C.; Koning, F. Nomenclature and Listing of Celiac Disease Relevant Gluten T-Cell Epitopes Restricted by HLA-DQ Molecules. *Immunogenetics* **2012**, *64*, 455–460, doi:10.1007/s00251-012-0599-z.
15. Juhász, A.; Belova, T.; Florides, C.G.; Maulis, C.; Fischer, I.; Gell, G.; Birinyi, Z.; Ong, J.; Keeble-Gagnère, G.; Maharajan, A.; et al. Genome Mapping of Seed-Borne Allergens and Immunoresponsive Proteins in Wheat. *Sci Adv* **2018**, *4*, eaar8602, doi:10.1126/sciadv.aar8602.
16. Altenbach, S.B.; Chang, H.-C.; Simon-Buss, A.; Mohr, T.; Huo, N.; Gu, Y.Q. Exploiting the Reference Genome Sequence of Hexaploid Wheat: A Proteomic Study of Flour Proteins from the Cultivar Chinese Spring. *Funct Integr Genomics* **2020**, *20*, 1–16, doi:10.1007/s10142-019-00694-z.
17. Sollid, L.M.; Khosla, C. Future Therapeutic Options for Celiac Disease. *Nat Clin Pract Gastroenterol Hepatol* **2005**, *2*, 140–147, doi:10.1038/ncpgasthep0111.
18. Kasarda, D.D. Can an Increase in Celiac Disease Be Attributed to an Increase in the Gluten Content of Wheat as a Consequence of Wheat Breeding? *J. Agric. Food Chem.* **2013**, *61*, 1155–1159, doi:10.1021/jf305122s.
19. Wang, J.; Luo, M.-C.; Chen, Z.; You, F.M.; Wei, Y.; Zheng, Y.; Dvorak, J. *Aegilops Tauschii* Single Nucleotide Polymorphisms Shed Light on the Origins of Wheat D-Genome Genetic Diversity and Pinpoint the Geographic Origin of Hexaploid Wheat. *New Phytol* **2013**, *198*, 925–937, doi:10.1111/nph.12164.
20. van Herpen, T.; Goryunova, S.; van der Schoot, J.; Mitreva, M.; Salentijn, E.; Vorst, O.; Schenk, M.; van Veelen, P.; Koning, F.; van Soest, L.; et al. Alpha-Gliadin Genes From the A, B, and D Genomes of Wheat Contain Different Sets of Celiac Disease Epitopes. *BMC Genomics* **2006**, *7*, 1, doi:10.1186/1471-2164-7-1.
21. Jouanin, A.; Boyd, L.; Visser, R.G.F.; Smulders, M.J.M. Development of Wheat With Hypoimmunogenic Gluten Obstructed by the Gene Editing Policy in Europe. *Front. Plant Sci.* **2018**, *9*, 1523, doi:10.3389/fpls.2018.01523.
22. Jouanin, A.; Gilissen, L.J.W.J.; Boyd, L.A.; Cockram, J.; Leigh, F.J.; Wallington, E.J.; van den Broeck, H.C.; van der Meer, I.M.; Schaart, J.G.; Visser, R.G.F.; et al. Food Processing and Breeding Strategies for Coeliac-Safe and Healthy Wheat Products. *Int. Food Res. J.* **2018**, *110*, 11–21, doi:10.1016/j.foodres.2017.04.025.
23. Morita, R.; Kusaba, M.; Iida, S.; Yamaguchi, H.; Nishio, T.; Nishimura, M. Molecular Characterization of Mutations Induced by Gamma Irradiation in Rice. *Genes Genet. Syst.* **2009**, *84*, 361–370, doi:10.1266/ggs.84.361.
24. Cockram, J.; Mackay, I.J.; O'Sullivan, D.M. The Role of Double-Stranded Break Repair in the Creation of Phenotypic Diversity at Cereal *VRN1* Loci. *Genetics* **2007**, *177*, 2535–2539, doi:10.1534/genetics.107.074765.
25. Kumar, R.; Kaur, A.; Pandey, A.; Mamrutha, H.M.; Singh, G.P. CRISPR-Based Genome Editing in Wheat: A Comprehensive Review and Future Prospects. *Mol. Biol. Rep.* **2019**, *46*, 3557–3569, doi:10.1007/s11033-019-04761-3.
26. Jouanin, A.; Gilissen, L.J.W.J.; Schaart, J.G.; Leigh, F.J.; Cockram, J.; Wallington, E.J.; Boyd, L.A.; van den Broeck, H.C.; van der Meer, I.M.; America, A.H.P.; et al. CRISPR/Cas9 Gene Editing of Gluten in Wheat to Reduce Gluten Content and Exposure—Reviewing Methods to Screen for Coeliac Safety. *Front. Nutr.* **2020**, *7*, 51, doi:10.3389/fnut.2020.00051.
27. Maeder, M.L.; Gersbach, C.A. Genome-Editing Technologies for Gene and Cell Therapy. *Mol. Ther.* **2016**, *24*, 430–446, doi:10.1038/mt.2016.10.
28. Karkute, S.G.; Singh, A.K.; Gupta, O.P.; Singh, P.M.; Singh, B. CRISPR/Cas9 Mediated Genome Engineering for Improvement of Horticultural Crops. *Front. Plant Sci.* **2017**, *8*, 1635, doi:10.3389/fpls.2017.01635.

29. Lieber, M.R. The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annu. Rev. Biochem.* **2010**, *79*, 181–211, doi:10.1146/annurev.biochem.052308.093131.
30. Bortesi, L.; Fischer, R. The CRISPR/Cas9 System for Plant Genome Editing and Beyond. *Biotechnol. Adv.* **2015**, *33*, 41–52, doi:10.1016/j.biotechadv.2014.12.006.
31. Liang, Z.; Chen, K.; Li, T.; Zhang, Y.; Wang, Y.; Zhao, Q.; Liu, J.; Zhang, H.; Liu, C.; Ran, Y.; et al. Efficient DNA-Free Genome Editing of Bread Wheat Using CRISPR/Cas9 Ribonucleoprotein Complexes. *Nat. Commun.***2017**, *8*, 14261, doi:10.1038/ncomms14261.
32. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **2012**, *337*, 816–821, doi:10.1126/science.1225829.
33. Rodrìguez-Rodrìguez, D.; Ramìrez-Solìs, R.; Garza-Elizondo, M.; Garza-Rodrìguez, M.; Barrera-Saldana, H. Genome Editing: A Perspective on the Application of CRISPR/Cas9 to Study Human Diseases (Review). *Int J Mol. Med.* **2019**, doi:10.3892/ijmm.2019.4112.
34. Parashar, D.; Geethadevi, A.; Aure, M.R.; Mishra, J.; George, J.; Chen, C.; Mishra, M.K.; Tahiri, A.; Zhao, W.; Nair, B.; et al. MiRNA551b-3p Activates an Oncostatin Signaling Module for the Progression of Triple-Negative Breast Cancer. *Cell Rep.* **2019**, *29*, 4389-4406.e10, doi:10.1016/j.celrep.2019.11.085.
35. Bak, R.O.; Dever, D.P.; Porteus, M.H. CRISPR/Cas9 Genome Editing in Human Hematopoietic Stem Cells. *Nat. Protoc.* **2018**, *13*, 358–376, doi:10.1038/nprot.2017.143.
36. Zhang, J.; Zhang, H.; Botella, J.R.; Zhu, J.-K. Generation of New Glutinous Rice by CRISPR/Cas9-Targeted Mutagenesis of the *Waxy* Gene in Elite Rice Varieties: CRISPR/Cas9 Create New Glutinous Rice Varieties. *J. Integr. Plant Biol.* **2018**, *60*, 369–375, doi:10.1111/jipb.12620.
37. Shan, Q.; Wang, Y.; Li, J.; Zhang, Y.; Chen, K.; Liang, Z.; Zhang, K.; Liu, J.; Xi, J.J.; Qiu, J.-L.; et al. Targeted Genome Modification of Crop Plants Using a CRISPR-Cas System. *Nat. Biotechnol.* **2013**, *31*, 686–688, doi:10.1038/nbt.2650.
38. Chen, K.; Wang, Y.; Zhang, R.; Zhang, H.; Gao, C. CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. *Annu. Rev. Plant Biol.* **2019**, *70*, 667–697, doi:10.1146/annurev-arplant-050718-100049.
39. Schindele, A.; Dorn, A.; Puchta, H. CRISPR/Cas Brings Plant Biology and Breeding into the Fast Lane. *Current Opinion in Biotechnology* **2020**, *61*, 7–14, doi:10.1016/j.copbio.2019.08.006.
40. Mohanta, T.; Bashir, T.; Hashem, A.; Abd_Allah, E.; Bae, H. Genome Editing Tools in Plants. *Genes* **2017**, *8*, 399, doi:10.3390/genes8120399.
41. Haurwitz, R.E.; Jinek, M.; Wiedenheft, B.; Zhou, K.; Doudna, J.A. Sequence- and Structure-Specific RNA Processing by a CRISPR Endonuclease. *Science* **2010**, *329*, 1355–1358, doi:10.1126/science.1192272.
42. Wiedenheft, B.; Sternberg, S.H.; Doudna, J.A. RNA-Guided Genetic Silencing Systems in Bacteria and Archaea. *Nature* **2012**, *482*, 331–338, doi:10.1038/nature10886.
43. Haft, D.H.; Selengut, J.; Mongodin, E.F.; Nelson, K.E. A Guild of 45 CRISPR-Associated (Cas) Protein Families and Multiple CRISPR/Cas Subtypes Exist in Prokaryotic Genomes. *PLoS Comput. Biol.* **2005**, *1*, e60, doi:10.1371/journal.pcbi.0010060.
44. Zhang, S.; Zhang, R.; Song, G.; Gao, J.; Li, W.; Han, X.; Chen, M.; Li, Y.; Li, G. Targeted Mutagenesis Using the Agrobacterium tumefaciens-Mediated CRISPR-Cas9 System in Common Wheat. *BMC Plant Biol* **2018**, *18*, 302, doi:10.1186/s12870-018-1496-x.
45. Deltcheva, E.; Chylinski, K.; Sharma, C.M.; Gonzales, K.; Chao, Y.; Pirzada, Z.A.; Eckert, M.R.; Vogel, J.; Charpentier, E. CRISPR RNA Maturation by Trans-Encoded Small RNA and Host Factor RNase III. *Nature* **2011**, *471*, 602–607, doi:10.1038/nature09886.
46. Jiang, W.; Marraffini, L.A. CRISPR-Cas: New Tools for Genetic Manipulations from Bacterial Immunity Systems. *Annu. Rev. Microbiol.* **2015**, *69*, 209–228, doi:10.1146/annurev-micro-091014-104441.
47. van der Oost, J.; Jore, M.M.; Westra, E.R.; Lundgren, M.; Brouns, S.J.J. CRISPR-Based Adaptive and Heritable Immunity in Prokaryotes. *Trends Biochem. Sci.* **2009**, *34*, 401–407, doi:10.1016/j.tibs.2009.05.002.
48. Garneau, J.E.; Dupuis, M.-È.; Villion, M.; Romero, D.A.; Barrangou, R.; Boyaval, P.; Fremaux, C.; Horvath, P.; Magadán, A.H.; Moineau, S. The CRISPR/Cas Bacterial Immune System Cleaves Bacteriophage and Plasmid DNA. *Nature* **2010**, *468*, 67–71, doi:10.1038/nature09523.
49. Mojica, F.J.M.; Diez-Villasenor, C.; Garcia-Martinez, J.; Soria, E. Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. *J Mol Evol* **2005**, *60*, 174–182, doi:10.1007/s00239-004-0046-3.
50. Gesner, E.M.; Schellenberg, M.J.; Garside, E.L.; George, M.M.; MacMillan, A.M. Recognition and Maturation of Effector RNAs in a CRISPR Interference Pathway. *Nat Struct Mol Biol* **2011**, *18*, 688–692, doi:10.1038/nsmb.2042.

51. Carte, J.; Wang, R.; Li, H.; Terns, R.M.; Terns, M.P. Cas6 Is an Endoribonuclease That Generates Guide RNAs for Invader Defense in Prokaryotes. *Genes Dev* **2008**, *22*, 3489–3496, doi:10.1101/gad.1742908.
52. Wiedenheft, B.; van Duijn, E.; Bultema, J.B.; Waghmare, S.P.; Zhou, K.; Barendregt, A.; Westphal, W.; Heck, A.J.R.; Boekema, E.J.; Dickman, M.J.; et al. RNA-Guided Complex from a Bacterial Immune System Enhances Target Recognition through Seed Sequence Interactions. *Proc Nat Acad Sci U S A.* **2011**, *108*, 10092–10097, doi:10.1073/pnas.1102716108.
53. Semenova, E.; Jore, M.M.; Datsenko, K.A.; Semenova, A.; Westra, E.R.; Wanner, B.; van der Oost, J.; Brouns, S.J.J.; Severinov, K. Interference by Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) RNA Is Governed by a Seed Sequence. *Proc Natl Acad Sci U S A* **2011**, *108*, 10098–10103, doi:10.1073/pnas.1104144108.
54. Miglani, G.S. Genome Editing in Crop Improvement: Present Scenario and Future Prospects. *J. Crop Improv.* **2017**, *31*, 453–559, doi:10.1080/15427528.2017.1333192.
55. Ricroch, A.; Clairand, P.; Harwood, W. Use of CRISPR Systems in Plant Genome Editing: Toward New Opportunities in Agriculture. *Emerging Top. Life Sci.* **2017**, *1*, 169–182, doi:10.1042/ETLS20170085.
56. Jiang, W.; Zhou, H.; Bi, H.; Fromm, M.; Yang, B.; Weeks, D.P. Demonstration of CRISPR/Cas9/SgRNA-Mediated Targeted Gene Modification in Arabidopsis, Tobacco, Sorghum and Rice. *Nucleic Acids Res.* **2013**, *41*, e188–e188, doi:10.1093/nar/gkt780.
57. Choulet, F.; Alberti, A.; Theil, S.; Glover, N.; Barbe, V.; Daron, J.; Pingault, L.; Sourdille, P.; Couloux, A.; Paux, E.; et al. Structural and Functional Partitioning of Bread Wheat Chromosome 3B. *Science* **2014**, *345*, 1249721, doi:10.1126/science.1249721.
58. Ling, H.-Q.; Zhao, S.; Liu, D.; Wang, J.; Sun, H.; Zhang, C.; Fan, H.; Li, D.; Dong, L.; Tao, Y.; et al. Draft Genome of the Wheat A-Genome Progenitor Triticum Urartu. *Nature* **2013**, *496*, 87–90, doi:10.1038/nature11997.
59. Wang, W.; Pan, Q.; He, F.; Akhunova, A.; Chao, S.; Trick, H.; Akhunov, E. Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. *CRISPR J.* **2018**, *1*, 65–74, doi:10.1089/crispr.2017.0010.
60. Kim, D.; Alptekin, B.; Budak, H. CRISPR/Cas9 Genome Editing in Wheat. *Funct. Integr. Genomics* **2018**, *18*, 31–41, doi:10.1007/s10142-017-0572-x.
61. Shan, Q.; Wang, Y.; Li, J.; Gao, C. Genome Editing in Rice and Wheat Using the CRISPR/Cas System. *Nat. Protoc.* **2014**, *9*, 2395–2410, doi:10.1038/nprot.2014.157.
62. Wang, Y.; Cheng, X.; Shan, Q.; Zhang, Y.; Liu, J.; Gao, C.; Qiu, J.-L. Simultaneous Editing of Three Homoeoalleles in Hexaploid Bread Wheat Confers Heritable Resistance to Powdery Mildew. *Nat. Biotechnol.* **2014**, *32*, 947–951, doi:10.1038/nbt.2969.
63. Gil-Humanes, J.; Wang, Y.; Liang, Z.; Shan, Q.; Ozuna, C.V.; Sánchez-León, S.; Baltes, N.J.; Starker, C.; Barro, F.; Gao, C.; et al. High-Efficiency Gene Targeting in Hexaploid Wheat Using DNA Replicons and CRISPR/Cas9. *Plant J* **2017**, *89*, 1251–1262, doi:10.1111/tpj.13446.
64. Liang, Z.; Chen, K.; Zhang, Y.; Liu, J.; Yin, K.; Qiu, J.-L.; Gao, C. Genome Editing of Bread Wheat Using Biolistic Delivery of CRISPR/Cas9 in Vitro Transcripts or Ribonucleoproteins. *Nat Protoc* **2018**, *13*, 413–430, doi:10.1038/nprot.2017.145.
65. Okada, A.; Arndell, T.; Borisjuk, N.; Sharma, N.; Watson-Haigh, N.S.; Tucker, E.J.; Baumann, U.; Langridge, P.; Whitford, R. CRISPR /Cas9-mediated Knockout of *Ms1* Enables the Rapid Generation of Male-sterile Hexaploid Wheat Lines for Use in Hybrid Seed Production. *Plant Biotechnol J* **2019**, *17*, 1905–1913, doi:10.1111/pbi.13106.
66. Howells, R.M.; Craze, M.; Bowden, S.; Wallington, E.J. Efficient Generation of Stable, Heritable Gene Edits in Wheat Using CRISPR/Cas9. *BMC Plant Biol* **2018**, *18*, 215, doi:10.1186/s12870-018-1433-z.
67. Zhang, S.; Zhang, R.; Gao, J.; Gu, T.; Song, G.; Li, W.; Li, D.; Li, Y.; Li, G. Highly Efficient and Heritable Targeted Mutagenesis in Wheat via the Agrobacterium Tumefaciens-Mediated CRISPR/Cas9 System. Int. J. Mol. Sci. **2019**, *20*, 4257, doi:10.3390/ijms20174257.
68. Abe, F.; Haque, E.; Hisano, H.; Tanaka, T.; Kamiya, Y.; Mikami, M.; Kawaura, K.; Endo, M.; Onishi, K.; Hayashi, T.; et al. Genome-Edited Triple-Recessive Mutation Alters Seed Dormancy in Wheat. *Cell Rep.* **2019**, *28*, 1362-1369.e4, doi:10.1016/j.celrep.2019.06.090.
69. Kamiya, Y.; Abe, F.; Mikami, M.; Endo, M.; Kawaura, K. A Rapid Method for Detection of Mutations Induced by CRISPR/Cas9-Based Genome Editing in Common Wheat. *Plant Biotechnol. J.* **2020**, *37*, 247–251, doi:10.5511/plantbiotechnology.20.0404b.
70. Raffan, S.; Sparks, C.; Huttly, A.; Hyde, L.; Martignago, D.; Mead, A.; Hanley, S.J.; Wilkinson, P.A.; Barker, G.; Edwards, K.J.; et al. Wheat with Greatly Reduced Accumulation of Free Asparagine in the Grain, Produced by CRISPR/Cas9 Editing of Asparagine Synthetase Gene *TaASN2*. *Plant Biotechnol. J.* **2021**, pbi.13573, doi:10.1111/pbi.13573.

71. Liu, H.; Wang, K.; Jia, Z.; Gong, Q.; Lin, Z.; Du, L.; Pei, X.; Ye, X. Efficient Induction of Haploid Plants in Wheat by Editing of TaMTL Using an Optimized Agrobacterium-Mediated CRISPR System. *J. Exp. Bot.* **2020**, *71*, 1337–1349, doi:10.1093/jxb/erz529.
72. Li, J.; Wang, Z.; He, G.; Ma, L.; Deng, X.W. CRISPR/Cas9-Mediated Disruption of TaNP1 Genes Results in Complete Male Sterility in Bread Wheat. *J Genet Genomics* **2020**, *47*, 263–272, doi:10.1016/j.jgg.2020.05.004.
73. Kamiya, Y.; Abe, F.; Mikami, M.; Endo, M.; Kawaura, K. A Rapid Method for Detection of Mutations Induced by CRISPR/Cas9-Based Genome Editing in Common Wheat. *Plant Biotechnolo.* **2020**, *37*, 247–251, doi:10.5511/plantbiotechnology.20.0404b.
74. Cui, X.; Balcerzak, M.; Schernthaner, J.; Babic, V.; Datla, R.; Brauer, E.K.; Labbé, N.; Subramaniam, R.; Ouellet, T. An Optimised CRISPR/Cas9 Protocol to Create Targeted Mutations in Homoeologous Genes and an Efficient Genotyping Protocol to Identify Edited Events in Wheat. *Plant Methods* **2019**, *15*, 119, doi:10.1186/s13007-019-0500-2.
75. Arndell, T.; Sharma, N.; Langridge, P.; Baumann, U.; Watson-Haigh, N.S.; Whitford, R. GRNA Validation for Wheat Genome Editing with the CRISPR-Cas9 System. *BMC Biotechnol* **2019**, *19*, 71, doi:10.1186/s12896-019-0565-z.
76. Zhang, R.; Liu, J.; Chai, Z.; Chen, S.; Bai, Y.; Zong, Y.; Chen, K.; Li, J.; Jiang, L.; Gao, C. Generation of Herbicide Tolerance Traits and a New Selectable Marker in Wheat Using Base Editing. *Nat. Plants* **2019**, *5*, 480–485, doi:10.1038/s41477-019-0405-0.
77. Zhang, Z.; Hua, L.; Gupta, A.; Tricoli, D.; Edwards, K.J.; Yang, B.; Li, W. Development of an Agrobacterium-Delivered CRISPR/Cas9 System for Wheat Genome Editing. *Plant Biotechnol J* **2019**, *17*, 1623–1635, doi:10.1111/pbi.13088.
78. Singh, M.; Kumar, M.; Albertsen, M.C.; Young, J.K.; Cigan, A.M. Concurrent Modifications in the Three Homeologs of Ms45 Gene with CRISPR-Cas9 Lead to Rapid Generation of Male Sterile Bread Wheat (Triticum aestivum L.). *Plant Mol Biol* **2018**, *97*, 371–383, doi:10.1007/s11103-018-0749-2.
79. Bhowmik, P.; Ellison, E.; Polley, B.; Bollina, V.; Kulkarni, M.; Ghanbarnia, K.; Song, H.; Gao, C.; Voytas, D.F.; Kagale, S. Targeted Mutagenesis in Wheat Microspores Using CRISPR/Cas9. *Sci. Rep.* **2018**, *8*, 6502, doi:10.1038/s41598-018-24690-8.
80. Zhang, Y.; Liang, Z.; Zong, Y.; Wang, Y.; Liu, J.; Chen, K.; Qiu, J.-L.; Gao, C. Efficient and Transgene-Free Genome Editing in Wheat through Transient Expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* **2016**, *7*, 12617, doi:10.1038/ncomms12617.
81. Upadhyay, S.K.; Kumar, J.; Alok, A.; Tuli, R. RNA-Guided Genome Editing for Target Gene Mutations in Wheat. *G3 (Bethesda)* **2013**, *3*, 2233–2238, doi:10.1534/g3.113.008847.
82. Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis Elegans. *Nature* **1998**, *391*, 806–811, doi:10.1038/35888.
83. Jorgensen, R.A.; Cluster, P.D.; English, J.; Que, Q.; Napoli, C.A. Chalcone Synthase Cosuppression Phenotypes in Petunia Flowers: Comparison of Sense vs. Antisense Constructs and Single-Copy vs. Complex T-DNA Sequences. *Plant Mol Biol* **1996**, *31*, 957–973, doi:10.1007/BF00040715.
84. Meyer, P.; Saedler, H. HOMOLOGY-DEPENDENT GENE SILENCING IN PLANTS. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **1996**, *47*, 23–48, doi:10.1146/annurev.arplant.47.1.23.
85. Gil-Humanes, J.; Pistón, F.; Hernando, A.; Alvarez, J.B.; Shewry, P.R.; Barro, F. Silencing of γ-Gliadins by RNA Interference (RNAi) in Bread Wheat. *J. Cereal Sci.* **2008**, *48*, 565–568, doi:10.1016/j.jcs.2008.03.005.
86. Agrawal, N.; Dasaradhi, P.V.N.; Mohmmed, A.; Malhotra, P.; Bhatnagar, R.K.; Mukherjee, S.K. RNA Interference: Biology, Mechanism, and Applications. Microbiol. Mol. Biol. Rev. **2003**, *67*, 657–685, doi:10.1128/MMBR.67.4.657-685.2003.
87. Obbard, D.J.; Gordon, K.H.J.; Buck, A.H.; Jiggins, F.M. The Evolution of RNAi as a Defence against Viruses and Transposable Elements. *Phil. Trans. R. Soc. B* **2009**, *364*, 99–115, doi:10.1098/rstb.2008.0168.
88. Chuang, C.-F.; Meyerowitz, E.M. Specific and Heritable Genetic Interference by Double-Stranded RNA in Arabidopsis Thaliana. *Proc Natl AcadSci USA.* **2000**, *97*, 4985–4990, doi:10.1073/pnas.060034297.
89. Fu, D.; Uauy, C.; Blechl, A.; Dubcovsky, J. RNA Interference for Wheat Functional Gene Analysis. *Transgenic Res* **2007**, *16*, 689–701, doi:10.1007/s11248-007-9150-7.
90. Travella, S.; Klimm, T.E.; Keller, B. RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat. *Plant Physiol.* **2006**, *142*, 6–20, doi:10.1104/pp.106.084517.
91. Gil-Humanes, J.; Piston, F.; Tollefsen, S.; Sollid, L.M.; Barro, F. Effective Shutdown in the Expression of Celiac Disease-Related Wheat Gliadin T-Cell Epitopes by RNA Interference. *Proc Natl Acad Sci USA.* **2010**, *107*, 17023–17028, doi:10.1073/pnas.1007773107.

92. Gil-Humanes, J.; Pistón, F.; Altamirano-Fortoul, R.; Real, A.; Comino, I.; Sousa, C.; Rosell, C.M.; Barro, F. Reduced-Gliadin Wheat Bread: An Alternative to the Gluten-Free Diet for Consumers Suffering Gluten-Related Pathologies. *PLoS ONE* **2014**, *9*, e90898, doi:10.1371/journal.pone.0090898.
93. Haro, C.; Villatoro, M.; Vaquero, L.; Pastor, J.; Giménez, M.; Ozuna, C.; Sánchez-León, S.; García-Molina, M.; Segura, V.; Comino, I.; et al. The Dietary Intervention of Transgenic Low-Gliadin Wheat Bread in Patients with Non-Celiac Gluten Sensitivity (NCGS) Showed No Differences with Gluten Free Diet (GFD) but Provides Better Gut Microbiota Profile. *Nutrients* **2018**, *10*, 1964, doi:10.3390/nu10121964.
94. Barrangou, R.; Birmingham, A.; Wiemann, S.; Beijersbergen, R.L.; Hornung, V.; Smith, A. van B. Advances in CRISPR-Cas9 Genome Engineering: Lessons Learned from RNA Interference. *Nucleic Acids Res.* **2015**, *43*, 3407–3419, doi:10.1093/nar/gkv226.
95. Hsu, P.D.; Lander, E.S.; Zhang, F. Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell* **2014**, *157*, 1262–1278, doi:10.1016/j.cell.2014.05.010.
96. Demirci, Y.; Zhang, B.; Unver, T. CRISPR/Cas9: An RNA-Guided Highly Precise Synthetic Tool for Plant Genome Editing. *J. Cell. Physiol.* **2018**, *233*, 1844–1859, doi:10.1002/jcp.25970.
97. Gilbert, L.A.; Larson, M.H.; Morsut, L.; Liu, Z.; Brar, G.A.; Torres, S.E.; Stern-Ginossar, N.; Brandman, O.; Whitehead, E.H.; Doudna, J.A.; et al. CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes. *Cell* **2013**, *154*, 442–451, doi:10.1016/j.cell.2013.06.044.
98. Boettcher, M.; McManus, M.T. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. *Mol Cell* **2015**, *58*, 575–585, doi:10.1016/j.molcel.2015.04.028.
99. Gilbert, L.A.; Horlbeck, M.A.; Adamson, B.; Villalta, J.E.; Chen, Y.; Whitehead, E.H.; Guimaraes, C.; Panning, B.; Ploegh, H.L.; Bassik, M.C.; et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **2014**, *159*, 647–661, doi:10.1016/j.cell.2014.09.029.
100. Smith, I.; Greenside, P.G.; Natoli, T.; Lahr, D.L.; Wadden, D.; Tirosh, I.; Narayan, R.; Root, D.E.; Golub, T.R.; Subramanian, A.; et al. Evaluation of RNAi and CRISPR Technologies by Large-Scale Gene Expression Profiling in the Connectivity Map. *PLoS Biol* **2017**, *15*, e2003213, doi:10.1371/journal.pbio.2003213.
101. Yin, Y.; Chory, J.; Baulcombe, D. RNAi in Transgenic Plants. *Curr. Protoc. Mol. Biol.* **2005**, *Chapter 26*, Unit 26.6, doi:10.1002/0471142727.mb2606s72.
102. Zischewski, J.; Fischer, R.; Bortesi, L. Detection of On-Target and off-Target Mutations Generated by CRISPR/Cas9 and Other Sequence-Specific Nucleases. *Biotechnol. Adv.* **2017**, *35*, 95–104, doi:10.1016/j.biotechadv.2016.12.003.
103. Verma, A.; Gatti, S.; Galeazzi, T.; Monachesi, C.; Padella, L.; Baldo, G.; Annibali, R.; Lionetti, E.; Catassi, C. Gluten Contamination in Naturally or Labeled Gluten-Free Products Marketed in Italy. *Nutrients* **2017**, *9*, 115, doi:10.3390/nu9020115.
104. Penagini, F.; Dilillo, D.; Meneghin, F.; Mameli, C.; Fabiano, V.; Zuccotti, G.V. Gluten-Free Diet in Children: An Approach to a Nutritionally Adequate and Balanced Diet. *Nutrients* **2013**, *5*, 4553–4565, doi:10.3390/nu5114553.
105. Makharia, G.K. Current and Emerging Therapy for Celiac Disease. *Front Med (Lausanne)* **2014**, *1*, 6, doi:10.3389/fmed.2014.00006.
106. Yoosuf, S.; Makharia, G.K. Evolving Therapy for Celiac Disease. *Front Pediatr* **2019**, *7*, 193, doi:10.3389/fped.2019.00193.
107. Wieser, H., Koehler, P., Folck, A., Becker, D.,Characterization of Wheat with Strongly Reduced α-Gliadin Content. *Gluten Proteins. AACC, St Paul, MN,* **2006**, 13–16.
108. Laursen, L. Erratum: Will Europe Toast GM Wheat for Gluten Sufferers? *Nat. Biotechnol.* **2016**, *34*, 1072, doi:10.1038/nbt1016-1072b.
109. Bawa, A.S.; Anilakumar, K.R. Genetically Modified Foods: Safety, Risks and Public Concerns—a Review. *J. Food Sci. Technol.* **2013**, *50*, 1035–1046, doi:10.1007/s13197-012-0899-1.
110. Weeks, D.P.; Spalding, M.H.; Yang, B. Use of Designer Nucleases for Targeted Gene and Genome Editing in Plants. *Plant Biotechnol J* **2016**, *14*, 483–495, doi:10.1111/pbi.12448.
111. Callaway, E. CRISPR Plants Now Subject to Tough GM Laws in European Union. *Nature* **2018**, *560*, 16–16, doi:10.1038/d41586-018-05814-6.
112. Belhaj, K.; Chaparro-Garcia, A.; Kamoun, S.; Nekrasov, V. Plant Genome Editing Made Easy: Targeted Mutagenesis in Model and Crop Plants Using the CRISPR/Cas System. *Plant Methods* **2013**, *9*, 39, doi:10.1186/1746-4811-9-39.
113. Ding, Y.; Li, H.; Chen, L.-L.; Xie, K. Recent Advances in Genome Editing Using CRISPR/Cas9. *Front. Plant Sci.* **2016**, *7*, doi:10.3389/fpls.2016.00703.

114. Weeks, D.P. Gene Editing in Polyploid Crops: Wheat, Camelina, Canola, Potato, Cotton, Peanut, Sugar Cane, and Citrus. In *Prog Mol Biol Transl Sci.*; Elsevier, 2017; Vol. 149, pp. 65–80 ISBN 978-0-12-811743-9.
115. Hall, N.J.; Rubin, G.; Charnock, A. Systematic Review: Adherence to a Gluten-Free Diet in Adult Patients with Coeliac Disease. *Aliment. Pharmacol. Ther.* **2009**, *30*, 315–330, doi:10.1111/j.1365-2036.2009.04053.x.