Chapter 12
Using Gene Editing Strategies for Wheat Improvement

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Abstract Despite wheat's global importance, it has trailed behind the other major cereals regarding genomic tools and resources as well as gene transformation. As each gene usually exists as two copies in the tetraploid durum wheat or as three in hexaploid bread wheat, it is very difficult assess gene function and improve important agronomic traits in polyploid wheat with traditional breeding methods. Recent advances allow researchers to use gene editing technologies in wheat which facilitates the opportunity to knockout or modify one, two or all three gene homoeologs simultaneously, which is important to clarify the function and contribution of gene copies in a specific phenotype or trait. CRISPR-Cas technology is now being used routinely for gene knockout. Technological advancement has been rapid within the field, and recently more advance and precise methods have been deployed such as cytidine base editing, adenosine base editing, and prime editing in wheat. Here we summarised gene editing strategies that are presently being applied for wheat improvement.

1 Introduction

Modern domesticated wheats are derivatives from ancient hybridisation events between ancestral progenitor species. The two most extensively cultivated wheats are the tetraploid durum or pasta wheat (*Triticum turgidum* ssp. *durum* L.) and hexaploid bread wheat (*Triticum aestivum* L.) Matsuoka and Nasuda [1]. Bread wheat is

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the major cultivated wheat species, while durum wheat accounts for about 5% of the total wheat production [2]. Both species have large genomes, durum ~12 and hexaploidy ~16 Gbp consisting mostly of repetitive elements. Within these polyploid species each gene usually exists as two copies in the tetraploid durum wheat or as three in hexaploid bread wheat. Homoeologous gene copies are usually highly conserved in gene structure and sequence among the subgenomes >95% [3]. Due to the polyploid nature of wheat, functional redundancy between homoeologs often occurs [4]. The traits controlled by recessive genes are particularly difficult to observe due to their multiple homeologs. This means that it may be necessary to manipulate all homoeologs and paralogs simultaneously to measure a phenotypic effect and this is very difficult to do it with traditional breeding methods [3]. A very low probability exists of the simultaneous mutation of genes in the A, B, and D genomes by natural processes or induced mutagenesis. Gene editing approaches gives the opportunity to knockout one, two or all three of the homoeologs of a gene, which is important to clarify the function and contribution of each homoeolog to a specific phenotype or trait.

Unrivalled in its geographic range of cultivation, wheat accounts for ~20% of the calorific value and ~25% of the daily protein intake of the world's population [5, 6]. In the 2020/2021 cropping season, over 770 million tons of wheat grain was harvested from over 220 million ha of arable land [7]. One of the three major cereal crops, along with maize (*Zea mays*) and rice (*Oryza sativa*), wheat has more influence on global food security than any other crop [5, 8]. Notwithstanding wheat's global importance, however, it has, until recently, trailed behind the other major cereals in regard to the development of genomic tools and resources for its improvement [2, 3]. One such tool is wheat transformation, a prerequisite for many CRISPR-Cas gene editing applications, which until lately had been languishing with low transformation efficiency ~5% and suffered with genotype dependence. Developments in open access robust transformation protocols and the use of morphological genes to improve regeneration, transformation efficiency and reduce genotype dependence have recently made outstanding improvements [9–11].

High quality DNA reference sequences of target genes are required for CRISPR gene editing, recent advances in sequencing technologies and bioinformatic tools have expediated wheat gene editing studies. Researchers are able to target multiple homoeoalleles simultaneously by CRISPR-Cas which enables the production of targeted mutations in all copies of a gene; therefore, the system holds great promise in the characterisation of genes endowing important agronomic traits in polyploid wheat. Furthermore, gene editing has been used to modify multiple genes simultaneously controlling different agronomic traits [12].

In wheat, CRISPR-Cas is being used for yield enhancement, improvement of grain quality, biofortification, development of resistance against diseases, and tolerance against abiotic factors (Table 12.1). The promising outcomes of the CRISPR-based multiplexing approach circumvent the constraint of targeting merely one gene at a time. Moreover, deployment of CRISPR-Cas variant systems such as cytidine base editing, adenosine base editing, and prime editing in wheat has been used to induce precise point mutations. The combination of these novel technologies

Table 12.1 Some agronomic traits improved by *CRISPR/Cas* in wheat

Target gene	Trait/Gene function	Editing type	Transformation method	Variety	References
TaLOX2	Expressed during grain development	Knockout	Particle bombardment	Bobwhite Kenong 199	[13]
Alpha-gliadin gene	Gluten protein	Knockout	Particle bombardment	BW208 and THA53, Don Pedro	[14]
TaGW2	Negative regulators of grain size and thousand grain weight	Knockout	Particle bombardment	Bobwhite Kenong 199	[15, 16]
TaGW7	Affects grain shape and weight	Knockout	Particle bombardment	Bobwhite	[17]
TaSPL	Affects grain size and number	Knockout	Particle bombardment	Fielder	[18]
TaARE1	Defective in N assimilation	Knockout	Particle bombardment	Zhengmai 7698	[19]
TaALS	A key enzyme in the biosynthesis of branched-chain amino acids and is known as an ideal herbicide tolerance	Knockout	Particle bombardment	Kenong 199 Kenong 9204	[20]
TaZip4-B2	Supress the level of homoeologous crossing over	Knockout	*A. tumefaciens*	Fielder Kronos	[21, 22]
TaCENH3α	*Paternal haploid induction Zygotic centromere formation*	Knockout	Particle bombardment	Fielder	[23]
TaSBEIIa	Increased amylose, resistant starch	Knockout	Particle bombardment	Zhengmai 7698 (ZM) and a spring wheat cv Bobwhite	[24]
TaASN2	Asparagine synthase gene	Knockout	Particle bombardment	Cadenza	[25]
WTAI-CM3 and WTAI-CM16	Involved in the onset of wheat allergies (bakers' asthma) and probably Non-Coeliac Wheat Sensitivity (NCWS)	Multiple knockout	Particle bombardment	Svevo	[26]
TaXip	Controlling the protein fractions, grain protein content, starch synthase, grain hardness, etc	Knockout	*A. tumefaciens*	Fielder	[27]

(continued)

Table 12.1 (continued)

Target gene	Trait/Gene function	Editing type	Transformation method	Variety	References
TaMLO	Durable and broad-spectrum resistance to powdery mildew	Knockout (RNP) Knockout	Particle bombardment	Bobwhite Kenong 199	[28, 29]
TaEDR1	Negatively regulates powdery mildew resistance in wheat	Knockout	Particle bombardment	Kenong 199	[30]
TaNFXL1	Represses trichothecene-induced defence responses and bacterial resistance	Knockout	Particle bombardment?	Fielder	[31]
TaHRC	Encodes a nuclear protein conferring FHB susceptibility	Knockout	Particle bombardment	Bobwhite	[32]
TaNFXL1	Represses trichothecene-induced defence responses and bacterial resistance	Knockout	Particle bombardment	Fielder	[31]
TaDREB2, TaDREB2	Stress-responsive transcription factor genes	Knockout	Protoplast	Chinese spring	[33]

addresses some of the most important limiting factors for sustainable and climate-smart wheat that should lead to the second "Green Revolution" for global food security.

Here we summarised advanced gene-editing tools to facilitate sustainable wheat production and use of these tools for the improvement of genetic traits related to the agronomic performance.

2 CRISPR-Cas Gene Editing

During the last two decades, there has been rapid development of genome-editing strategies that make it possible to directly target regions of genes in a DNA sequence-specific manner. Site-directed nucleases (SDNs)-based gene editing technologies considerably enhance the precision of gene modification in plants [34]. This set of tools, comprising zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats associated protein (CRISPR-Cas) [35, 36], allow to repress or activate gene expression, modification of gene function, or create gene knockouts, mediating the targeted manipulation of DNA sequences [37]. Gene editing involves the introduction of targeted DNA double-strand breaks (DSBs) at the specific targeted site within a gene by using an engineered nuclease, which induces cellular DNA repair

mechanisms. Once a DSB is induced repair pathways, such as, the non-homologous end-joining (NHEJ) mechanism or homologous recombination (HR) pathway can repair the induced DSBs introducing simultaneously the desired modifications at the target locus [38]. Due to its easiness, accuracy and effectiveness, along with its ability to produce transgene-free, gene edited crops, CRISPR-Cas rapidly diffused as the most used site-directed nucleases (SDNs)-based gene editing technology. The CRISPR-Cas system is usually introduced into plants as transgenes, however, in the following generation the transgene can be segregated away, leaving a transgene-free plant containing the desired mutations. Zhang et al. [13] developed a highly efficient transient expression-based gene-editing system for producing transgene-free and homozygous wheat mutants in the T0 generation. Liang et al. [40] reported an efficient gene edited method to produce transgene-free plants by CRISPR/Cas9 ribonucleoproteins (RNPs) in wheat [30, 39, 40].

In nature, CRISPR-Cas exists as a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements such as bacterial phage. Biotechnologists have harnessed and modified this system to enable gene editing applications in multiple species, including plants. There are over 30 different CRISPR-Cas systems naturally occurring that have been reported [41], however, the majority of gene editing applications are performed by CRISPR-Cas9 based systems, followed closely by CRISPR-Cas12a, previously known as CpfI. The Cas9 nuclease was first thought to make a blunt DSB of DNA, three base pairs upstream of the PAM, however, evidence strongly suggests that Cas9 leaves a single nucleotide 5′ overhang [42]. Cas12a on the other hand leaves 5 bp staggered overhang at the opposite end of the PAM motif making it more favoured for applications such as gene targeting [43] or recently reported disruption of *cis*-regulatory elements within promoter regions [44].

Since the first report of CRISPR-Cas technology being used for gene editing, technological advancement has been rapid within the field. There are experimentally derived protocols for the selection of sgRNA targets, construct assembly, and screening analysis for genome editing in hexaploid wheat such as Smedley et al. [45]. Although, the majority of reports are for gene knockout, researchers are now able to perform targeted base changes through base editing, rewrite small length of DNA using prime editing, insertion of DNA via gene targeting, upregulation or suppression of gene expression as well as multi-gene knockouts.

3 Multiplex Gene Editing

CRISPR-Cas based gene editing technologies enable the easy modification of two or more specific DNA loci in a genome with high precision. These tools have greatly increased the feasibility of introducing desired changes in specific but different genes, resulting in the development of new plant genotypes carrying multiple mutations in a single generation. There are three main strategies to produce multiple single-guide RNAs (sgRNAs), the conventional multiplex system with separate U3

or U6 promoters driving individual sgRNAs, the tRNA-processing system [46] and the ribozyme-processing system [47]. The polycistronic tRNA-sgRNA system consists of the sgRNAs linked together by tRNA sequences, the guides are transcribed in a single transcript. The tRNA sequences are then recognized and processed by endogenous RNases that excise the individual sgRNAs from the transcript [46]. The ribozyme system consists of a single transcript of multiple sgRNA, where individual sgRNAs are flanked by self-cleaving ribozyme sequences such as the hammerhead ribozyme (HH) and the hepatitis delta virus (HDV) ribozyme, [47, 48]. Transcripts are cleaved by the cis-acting ribozymes post-transcriptionally. Wang et al. [49] deployed a multiplexing gene editing approach based on the tRNA system in wheat to target *TaGW2*, *TaLpx-1,* and *TaMLO* genes, simultaneously. A similar tRNA approach was used in durum wheat cultivar Svevo by Camerlengo et al. [26] to edit the α-amylase/trypsin inhibitor subunits WTAI-CM3 and WTAI-CM16 in the grain to reduce allergen proteins. Abdallah et al. [50] created *TaSal1* mutants using this multiplex system to address drought tolerance in wheat. The three main multiplex CRISPR-Cas systems for simultaneous gene editing at 8 target sites in bread wheat were tested by Li et al. [51]. The ribozyme and tRNA systems were found to be more effective at gene editing than the conventional multiplex system with individual promoter driving individual guides [51].

4 Base Editing

Base editing enables the generation of targeted point mutations without DSBs, DNA donor templates, or the reliance of the homologous repair (HR) pathway [52, 53]. Base editors consist of a DNA deaminase fused to a catalytically impaired Cas nuclease such as deactivated Cas9 (dCas9) or a Cas9 nickase (nCas9). The Cas-deaminase fusion is guided to the target site by the guide RNA, where a single stranded DNA R-loop is formed allowing access for the DNA deaminase [54]. Where the deaminase is fused to SpCas9 variants, the 'activity window' for base editing spans approximately protospacer positions 4–8 (position 1 being the first nucleotide of the protospacer, the PAM being at positions 21–23). There are two main classes of base editors which have been developed so far: cytosine base editors (CBEs), which catalyse the conversion of C/G base pairs to T/A base pairs; and adenine base editors (ABEs), which catalyse A/T-to-G/C conversions [52, 53]. Therefore, CBEs and ABEs can facilitate four possible transition mutations (C → T, A → G, T → C, G → A). Improvements in base editor efficiency such as the inclusion of uracil glycosylase inhibitor proteins (UGI) typically CBEs such as BE3 [53] and optimisation of linker sequences Komor et al. [55] have substantially increased base editing yield, extensively reviewed in Anzalone et al. [54]. Zong et al. [56] used a CBE in both protoplasts and regenerated rice, wheat, and maize plants at frequencies of up to 43.48 in rice%. Li et al. [57], from same research group, described optimisation of an ABE for application in plant systems, demonstrating its high

efficiency in creating targeted point mutations at multiple endogenous loci in rice and wheat. Han et al. [58] compared two ABEs, ABE7.1 and the new ABE8e containing a high-activity adenosine deaminase TadA8e, both were codon optimised for wheat. To aid nuclear localisation Bipartite-SV40-Nuclear-Localization-Signals (bpNLS) were added at the N-terminus of TadA* and also a bpNLS followed by a nucleoplasmin NLS (npNLS) at the C-terminus of SpCas9n. Calling them wheat high-efficiency ABEs (WhieABE7.1 and WhieABE8e), it was found in the study that WhieABE8e out performed WhieABE7.1 when targeting 5 wheat tubulin alleles.

5 Prime Editing

Prime-editing technology enables targeted insertions, deletions and all 12 types of point mutation without requiring double-strand breaks or donor DNA templates. It expands the scope and capabilities of directly targeting and modifying genomic sequences. Prime editing is achieved by the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) fused to the C-terminus of Cas9 H840A nickase (nCas9) together with engineered prime editing guide RNA (pegRNA). Primer Editors (PEs) directly copy the desired genetic information from the pegRNA into the target genomic locus [59]. The pegRNA contains two parts: a primer binding site (PBS) and an RT template (RTT) and guides the nickase to the target site by homology to a genomic DNA locus. nCas9 recognizes and nicks the nontarget DNA strand of the target site and releases a single-strand DNA, and the PBS then hybridises with the released DNA and serves as a primer for reverse transcription. The desired edits encoded by the RTT are then reverse transcribed and transferred to the nontarget DNA strand, generating a DNA flap that is subsequently incorporated into the target site by DNA repair [60].

Initially, PE1, PE2, PE3 and PE3b were characterized by Anzalone et al. [54] who developed the technology in mammalian cells. PE1 is a fusion of Cas9 nickase to the wild-type M-MLV RT. PE2 substitutes for the wild-type M-MLV RT an engineered pentamutant M-MLV RT. PE3 combines the PE2 fusion protein and pegRNA with an additional sgRNA that targets the non-edited strand for nicking. A variant of the PE3 system called PE3b uses a nicking sgRNA that targets only the edited sequence, resulting in decreased levels of indel products by preventing nicking of the non-edited DNA strand until the other strand has been converted to the edited sequence [54].

Lin et al. [61] compared PE2, PE3, and PE3b in wheat protoplasts and rice, they produced a wide variety of edits at genomic sites in rice and wheat. Prime-edited efficiencies up to 21.8% were reported for regenerated rice plants [61]. An N-terminal RT–Cas9 nickase fusion PE and multiple synonymous nucleotide substitutions introduced into the RT template increased the average efficiency of prime editing in rice to 24.3% Xu et al. [59]. An engineered plant prime editor (ePPE) was

developed by removing the ribonuclease H domain from M-MLV RT and incorporating a viral nucleocapsid protein with nucleic acid chaperone activity. This enhanced the frequency of various targeted modifications, including base substitutions, insertions (34 bp) and deletions (90 bp) by an average of 5.8-fold in rice and wheat protoplast compared to PE2 [62]. An online design tool (PlantPegDesigner) was developed for designing efficient strategies for PE in plants, including dual-pegRNA designs [60]. The pegRNA primer-binding site length, RT template length and nicking sgRNA position effects editing frequencies in plants [60, 61].

6 Other Strategies

Strategies have been developed to deliver CRISPR system components into plant germline or meristematic cells that achieves genotype-independent editing. Plant RNA virus-based vector systems can deliver gene editing reagents into plant leaves. Based on this approach an engineered *Barley stripe mosaic virus*–based sgRNA delivery vector (BSMV-sg) was used by Li et al. [63] to perform heritable gene editing in Cas9 expressing transgenic wheat plants. The progeny in the next generation had editing at frequencies ranging from 12.9% to 100% in three different wheat varieties, and 53.8–100% of edited plants were virus free. The group achieved multiplex editing in the progeny using a pool of BSMV-sg vectors harbouring different sgRNAs and were able to generate Cas9-free wheat mutants by crossing BSMV-infected Cas9-transgenic wheat pollen with wild-type wheat.

Other delivery methods were developed in wheat. Nanomaterials have emerged as a promising candidate for delivery of genetic cargoes to intact plant cells. Functionalised high-aspect-ratio carbon nanotube (CNT) nanoparticles (NPs) have been successfully used in wheat leaves for efficient DNA delivery [64]. The use of nanomaterials for GE studies has yet to be reported.

Liu et al. [65] developed an *in planta* particle bombardment (iPB) method which has increased process efficiency since no culture steps are required to create stably genome-edited wheat plants. The biolistic delivery of gold particles coated with plasmids expressing CRISPR-Cas9 components designed to target *TaQsd1* were bombarded into the embryos of imbibed seeds with their shoot apical meristem (SAM) exposed. A total of 2.51% of the bombarded plants (cv. "Haruyokoi," spring type) carried mutant alleles in the tissue. The method utilised transient expression of CRISPR-Cas and no detectable transgene integration was identified. Kumagai et al. [66] reported an iPB-ribonucleoprotein (RNP) method which represents an alternative approach for creating genome-edited wheat varieties with an editing efficiency comparable to the iPB-DNA method. Since no DNA is used, and therefore no transgene integration occurs, the iPB-RNP method has the potential for use in modern agricultural applications and commercialisation.

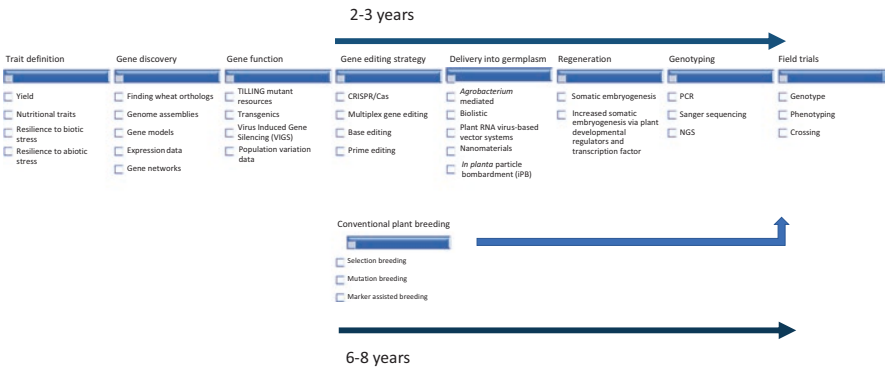

Fig. 12.1 Pathway for the improvement of genetic traits in wheat by gene-editing and conventional plant breeding

7 Recent Applications of Gene Editing in Wheat

Since the domestication of cereal crops, farmers selected plants with favorable agronomic traits that led to increased crop yield and performance. The introduction of semi-dwarf varieties and agrochemicals, starting from the second half of the last century, led to a leap forward in determining substantial increases in grain yield. In the modern agrifood context, crop yield remains one of the major traits to be improved to meet growing demands for food production and climate change effects. More recently, breeders and scientists also focused on the quality values of wheat grains to improve technological end-use quality of wheat flour and the nutritional value of derived foods in addition to the aim to produce safer genotypes with reduced toxic, immunogenic and antinutritional compounds.

To date, gene-editing tools have been demonstrated to extensively contribute to the study and improvement of genetic traits related to the agronomic performance and end-use quality of wheat, decreasing the time for the generation of new genotypes harboring precise mutations that can enhance grain yield and quality (Fig. 12.1).

8 Gene Editing for Grain Quality Improvement

Grain quality refers to the technological behavior of flours and doughs obtained from wheat as well as to internal quality factors such as nutrients and bioactive compounds. In recent years aspects related to nutritional and healthy values have gained more prominence since several disorders have been associated with wheat derived food consumption. In this respect, gene editing tools provide the ability to fine regulate the chemical composition of wheat kernels modulating biosynthetic pathways of major components such as protein, starch and lipids as well as offer the

opportunity to produce wheat with improved nutritional value, in which minor components play a predominant role.

Starch modifications in wheat were widely explored in the last two decades leading to wheat genotypes with diverse amylose/amylopectin ratio that influence not only the rheological behavior of doughs but also the nutritional value of derived foods. In general, starch contains two major glucose polymers, amylose and amylopectin, which differ in the degree of polymerization (DP) of glucan chains and in the frequency of branches. In wheat endosperm, starch consists of approximately 70–80% amylopectin and 20–30% amylose. Increasing the amount of amylose at the expense of amylopectin, the fraction of starch not digested and absorbed in the gastrointestinal trait (referred to as resistant starch) can be enhanced with a consequent beneficial effect on human health, correlated to blood glycemic index after wheat derived food consumption. Li et al. [24], using CRISPR-Cas9 in bread wheat, generated a series of transgene-free mutant lines with partial or triple-null *TaSBEIIa* alleles, an isoform of starch-branching enzymes. The triple-null lines (aabbdd) showed significantly increased amylose content, resulting in higher content of resistant starch, protein and soluble pentosan whereas a slight decrease of total starch was observed. On the other hand, they observed a series of pleiotropic effects related to plant growth and grain morphology traits besides negative effects on baking qualities of derived flours. Decreased plant height and tiller number, lower grain length, width and lower grain number associated with reduced thousand grain weight were observed in mutant lines compared to the control. In addition, the decrease in amylopectin content, generally recognized to be related to good end-use quality, strongly influenced the viscosity parameters and negatively affected the rheological proprieties of doughs.

Dough stability time (ST) and SDS-sedimentation values (SV) are two of the major quality parameters used to determines the final quality of bread wheat flours. These two parameters are mostly influenced by protein content and composition and are positively correlated to dough rheological properties. Sun et al. [27] reported for the first time the effect of proteinaceous inhibitors of endo-xylanases. In particular, they found a QTL for dough stability time and SDS-sedimentation value on chromosome 6A (*QSt/Sv-6A-2851*) and identified the *xylanase inhibitory protein* (*TaXip*) gene as the principal genetic component of the QTL producing variation in the above-mentioned parameters. They validated all the three homeoalleles (*TaXip-6A*, *TaXip-6B*, and *TaXip-6D*) producing CRISPR-Cas9 knock-outs, albeit they produced only two mutant genotypes (aaBBDD and AAbbdd). The SDS-sedimentation value was significantly higher in both mutant lines compared to the control whereas the stability time value was significantly higher only for aaBBDD genotype compared to the control. Based on these results and on the observation of homoeologous gene expression in the grain at the later stage of grain development, the authors claim that *TaXip-6A* has a greater effect on quality parameters compared to *TaXip-6B* and *TaXip-6D*.

Zhang et al. [15] observed an increase in the SDS sedimentation volume of bread wheat lines in which they edited *TaGW2* homoeologous genes. Grain protein

content and flour protein content resulted considerably elevated in all the mutant lines, particularly in AAbbDD and AAbbdd genotypes. Glutenins and gliadins were also increased. Otherwise, they observed that the grains of double and triple mutants were morphologically wrinkled compared to the control plants and single mutants.

Grain quality defects such as late maturity amylase (LMA) and pre-harvest sprouting (PHS) are correlated with low Hagberg falling number (FN), which is considered an indicator of the amount of sprout damage caused by enzyme activity (α-amylase) producing the decrease of dough viscosity. PHS determines the germination of grains while they are still on the spike causing significant decrease of grain quality. Abe et al. [67] produced loss-of function mutations of *TaQsd1* gene that controls seed dormancy in wheat and barley resulting in longer seed dormancy. They generated all triple homozygous transgene-free genotypes (AABBDD, aaBBDD, AAbbDD, AABBdd, aabbDD, aaBBdd, AAbbdd, and aabbdd) but only the triple mutant (aabbdd) showed significantly different germination rates and reduced PHS.

The consumption of wheat derived foods is associated to the increasing incidence of wheat related pathologies such as Coeliac Disease (CD), allergies and Non-Coeliac Wheat Sensitivity (NCWS) but also food processing of wheat flour can lead to the formation of antinutritional and toxic compounds.

Free asparagine is converted to acrylamide, a carcinogenic contaminant, during high-temperature processing of food made from wheat flour. Raffan et al. [25] knocked out the asparagine synthase gene, *TaASN2,* in bread wheat genotypes to reduce the concentration of free asparagine in the grain. They observed an almost total reduction of free asparagine concentrations in the grain of triple-null mutants genotypes (aabbdd). In contrast, an increase in free glutamine, glutamate and aspartate was found in all the edited lines. Raffan et al. [68] used the low asparagine edited lines for the first field trial of genome-edited wheat lines in Europe.

The main triggering factors of CD are prolamins, glutenins and gliadins that are proteins contained in wheat grain endosperm and responsible for the gluten matrix formation. Sánchez-León et al. [14] used the CRISPR/Cas9 technology to reduce the content of α-gliadins in wheat kernels. Since α-gliadins represent a large protein family with high sequence homology among members, they produced multiplex editing of gliadins targeting conserved regions among the gene family members. Although pleiotropic effects on the other gliadins classes (ω- and γ-) were observed, the mutant lines could be used to produce low-gluten wheat derived foods.

Other endosperm protein families such as a-amylase/trypsin inhibitors (ATI), which are structural and metabolic proteins involved in plant defense mechanisms, can trigger the onset of wheat allergies and NCWS. Camerlengo et al. [26] used a multiplexing strategy to edit the ATI subunits WTAI-CM3 and WTAI-CM16 in durum wheat producing transgene-free wheat lines with a reduced amount of ATI. The mutant lines completely lacked target ATI subunits resulting in a decrease of their allergenic potential.

9 Gene Editing for Grain Yield

One of the major goals of wheat breeding remains the improvement of yield. It is related to both the agronomic performance of crops as well as to environmental conditions. The constitution of semi-dwarf wheat varieties along with the introduction of chemicals and modern agronomic practice lead to a substantial increase of grain yield starting from the second half of last century.

Nowadays, a second "Green-Revolution" is required to face the global food demand due to the rapid increase of world population and to the worsening of climate change.

Grain morphology traits such as grain weight (GW), grain width (GWH) and grain length (GL) constitute a breeding target to enhance grain productivity. The knock-out of *TaGW2* homoeologous genes in two bread wheat cultivars had higher GWH and GL values and resulted in increased thousand grain weight (TGW) values of mutant lines compared to the control [15, 16]. Mutations in the homoeologous *TaGW2* genes had dosage-dependent effects on phenotypes in both bread wheat cultivar; although each homoeologous gene had different effect in the two genotypes, significant changes of TGW were associated with changes in gene dosage rather than with specific combination of mutated alleles.

TaGW7 encodes a TONNEAU1-recruiting motif (TRM) protein that affects grain morphology and weight in wheat and other cereal species. Wang et al. [17] demonstrated, by editing *TaGW7* in bread wheat, that mutations in the homoeologous genes of the B and D genomes increased the GWH and TGW but reduced the GL. They produced single (AABBdd) and double mutants (AAbbdd) with wider and shorter grains compared to the control, and *TaGW7-D1* seems to contribute at a greater extent to the phenotypic effects affecting grain size. Similar to *TaGW2*, *TaGW7* had dosage-dependent effects on phenotypes.

Another important target for improving grain yield is the *SQUAMOSA promoter-binding protein-like* (SPL) genes encoding for transcription factors that regulates a plethora of plant developmental and yield-related traits. The *SPL* family members are often negatively regulated by micro-RNA 156/157 (miR156). Gupta et al. [18] identified the microRNA 156 recognition elements (MRE) in the 3′-untraslated region of the *TaSPL13* gene and, using CRISPR-Cas9, they generated mutations in the three homoeologous genes in bread wheat. Mutations in MRE led to a higher expression of *TaSPL13* which produced a decrease in flowering time, tiller number and plant height but increased grain size and number.

To improve grain yield farmers are used to applying nitrogen fertilizers, but this type of agriculture practice leads to aggravating environmental pollution and ecological deterioration. Furthermore, most of the modern wheat varieties show low Nitrogen Use Efficiency (NUE) and absorb less than 40% of the supplied nitrogen. Zhang et al. [19] isolated and characterized the *abnormal cytokinin response1 repressor1* (*TaARE1*) gene in a Chinese winter wheat cultivar and then used CRISPR-Cas9 to generate a series of transgene-free mutant lines either with partial or triple-null *TaARE1* alleles. Loss of function mutations in this gene result in

delayed senescence, enhanced NUE and increased grain yield under normal field conditions. All the edited lines showed enhanced tolerance to N starvation with the AABBdd and aabbDD genotypes exhibiting significantly improved NUE without growth penalties compared to the control that results in increased TWG.

W eed competition is related to yield loss in wheat cultivation. Zhang et al. [20] generated transgene-free wheat germplasm by base editing the *acetolactate synthase* (*ALS*) and *acetylcoenzyme A carboxylase* genes. TaALS is a key enzyme in the biosynthesis of branched-chain amino acids and is known as an ideal herbicide tolerance target in wheat. Edited wheat lines, harboring different point mutations, were evaluated for the tolerance to multiple herbicides; homozygous mutants with four or six edited alleles showed enhanced tolerance to herbicides and grew normally whereas control plants died in few weeks after herbicide application. This strategy could be directly applied to produce wheat varieties tolerant to herbicides but also can be exploited as selection marker in wheat transformation and in vitro regeneration.

A number of important agronomic traits have been targeted by gene editing in wheat, although obtaining new high-yielding cultivars also resistant to biotic and abiotic stresses remains the main objective of most plant breeding programs. In the next sections, gene editing system successfully used for gene editing in wheat are reported.

10 Gene Editing for Biotic Stress Resistance

Pest and disease, among biotic constraints, are estimated to determine 21.5% of current yield losses [69]. Modern agriculture relies on chemical compounds to avoid and prevent yield losses due to fungal diseases, but the extensive application of such chemicals severely affects both human health and the environment. So far, the development of fungus-resistant wheat cultivars has been a noteworthy goal in wheat breeding programs. Gene editing in wheat has targeted several genes for improvement of resistance against diseases caused by fungi, specifically *Blumeria graminis* and *Fusarium graminearum*.

The first successful experiment using the CRISPR-Cas system in wheat was reported by Shan et al. [70], who edited the *MLO* gene, which encodes for the MILDEW-RESISTANCE LOCUS (MLO) protein. The authors reported a mutation frequency of *TaMLO* in protoplasts of 28.5%. MLO has a negative resistance function, thereby causing susceptibility to powdery mildew in plants expressing this gene [71]. Powdery mildew diseases are caused by *Blumeria graminis* f. sp. *tritici* and result in significant wheat yield losses. As knockout of the *TaMLO* leads to disease resistance, this gene was an ideal target for RNA-guided Cas9 knockouts to improve powdery mildew tolerance. The approach was successfully demonstrated by Wang et al. [28], who simultaneously targeted the three homoeoalleles of *TaMLO* in hexaploid bread wheat with both CRISPR-Cas9 and TALEN technologies using particle bombardment, which resulted in powdery mildew resistant plants. The

mutation frequency of regenerated *TaMLO*-edited wheat (5.6%) was similar for both editing methods.

More recently, Zhang et al. [30] demonstrated an improved powdery mildew resistance in wheat by simultaneously modifying the three homoeologous of *TaEDR1* by CRISPR-Cas9 gene editing. The *enhanced disease resistance1* (*EDR1*) gene is a negative regulator of resistance to powdery mildew in Arabidopsis Frye et al. [72]. The authors targeted a highly conserved region within the coding sequence of *TaEDR1*, obtaining homoeologous stable *Taedr1* mutations inherited in the T1 generation with a transmission rate of 97–100%. *Taedr1* plants were resistant to powdery mildew and did not show mildew-induced cell death.

Several studies have also been focused on *Fusarium gramineaurm* resistance, a fungus causing one of the most detrimental diseases in wheat. The lipoxygenase genes, *TaLpx* and *TaLox*, have been found to be good targets for gene editing resistance to Fusarium. These enzymes play a key role in the jasmonic acid-mediated defense responses in plants by catalyzing the hydrolysis of polyunsaturated fatty acids and then activating oxylipins biosynthesis. Nalam et al. [73] found that *TaLpx-1* gene silencing resulted in resistance to *Fusarium graminearum* in wheat. Shan et al. [16, 70] respectively edited *TaLpx* and *TaLox* genes in protoplasts, detecting a mutation frequency of 9% and 45%. Further studies by Zhang et al. [13] allowed to obtain wheat plants with mutated *TaLOX* with a frequency of 9.5%, of which homozygous mutants accounted for 44.7%.

Fusarium head blight (FHB) resistance was also investigated by targeting three wheat genes including an ABC transporter (*TaABCC6*), the nuclear transcription factor X box-binding like1 (*TaNFXL1*) both associated with FHB susceptibility, and a gene encoding a nonspecific lipid transfer protein (nsLTP), *TansLTP9.4*, which correlates with FHB resistance. PCR amplicons from protoplasts transformed with editing constructs were sequenced, showing that the three genes had been successfully edited with efficiencies of up to 42.2% [74]. Another target for Fusarium resistance was reported by Su et al. [32], who identified HISTIDINE RICH CALCIUM-BINDING PROTEIN (*TaHRC*) as a quantitative trait locus (QTL) responsible for resistance to Fusarium head blight. By cloning and sequencing a candidate gene they found a single deletion on the B genome homeolog, which was sufficient to determine resistance to Fusarium head blight. This result was confirmed by CRISPR-Cas9 approach targeting the *TaHRC* homeolog from the B subgenome, which caused frameshift mutations resulting in more than 40% reduction in fusarium head blight disease in wheat. This study showed that it is not always necessarily to knocked out all three homeologs in wheat, and in some cases the inactivation of one single homeolog is enough to successfully induce disease resistance.

More recently, Brauer et al. [31] followed a different approach to generate mutants resistant to Fusarium head blight. They focused on the NUCLEAR TRANSCRIPTION FACTOR, X-BOX BINDING 1-LIKE (NFXL1), a transcription factor used by Fusarium to repress defense responses upon infection, which is present as two copies on each of the wheat subgenomes. The authors showed that NFXL1 downregulation via RNAi in barley confers partial resistance upon

infection. Wheat NFXL1 knockout mutants with edits in all six homoeologous in the T1 generation were then obtained by designing sgRNA pair targeting all six loci simultaneously (one homeolog remained in heterozygous state), thus showing an increased Fusarium head blight resistance, similarly to that obtained through RNAi.

11 Gene Editing for Abiotic Stress Resistance

Using CRISPR-Cas to design resilience to abiotic stress in an era of global warming and extreme weather events is a justifiable aim. Abiotic stresses such as drought, extreme temperatures and salinity affect plant growth, survival, reproducibility and production, furthermore, it was shown that abiotic stresses modulate epigenetic changes in plants [75].

Genome engineering represent an effective approach to increase plant growth in response to abiotic stress in wheat, especially considering that abiotic stress-associated genes involved in cellular and molecular responses can easily be targeted with the available gene editing tools. So far, only few studies have shown the application of gene editing to improve abiotic stress response. CRISPR-Cas9 gene editing was successfully used in transient transformation in wheat protoplast. Two important abiotic stress-responsive transcription factor genes were targeted: *TaDREB2*, (wheat dehydration responsive element binding protein 2) and *TaERF3*, a wheat ethylene responsive factor 3 [33]. The results suggested that CRISPR-Cas gene editing has huge potential for manipulation of wheat genome to improve stress tolerance and obtain better crop performances, despite the challenging ploidy level of wheat. Abdallah et al. [50] used a multiplex gene editing approach to knockout the five active homologous gene copies of *TaSal1* in wheat variety Giza 168. In the primary transgenic plants 34% showed editing, these edits were heritable, and, in the progeny, five lines were identified with all five copies of *TaSal1* edited. Young leaves of edited *TaSal1* lines showed closed stomata, increased stomata width and increase in the size of the bulliform cells. *TaSal1* edited seedlings germinated and grew better on media containing polyethylene glycol than wildtype seedlings [50].

Further research and breeding programs will still be necessary to better elucidate the genetic and physiological bases of metabolic and signaling pathways of stress tolerance mechanisms. The identification of gene regulatory networks involved in stress responses and their targeting by gene editing tools will allow the development of new stress tolerant and high yielding varieties.

12 Summary

Recent advances in the production of high-quality genome sequences and efficient genotype- independent transgenic methods, developments in CRISPR-Cas based gene editing tools bring functional genomics and rational design-based molecular

breeding of polyploid wheat to unlock its hidden potential. Gene edited transgene free wheat has a critical role in addressing environmental issues while promoting sustainable agriculture and global food security. Gene editing is just one of the methods advancing wheat breeding programs and supporting wheat biology, which can be used alongside more conventional commercial breeding methods for delivering sustainability.

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