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REVIEW

Advances in bread wheat production through CRISPR/Cas9 technology: a comprehensive review of quality and other aspects

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Abstract

Main conclusion **This review provides a comprehensive overview of the CRISPR/Cas9 technique and the research areas of this gene editing tool in improving wheat quality.**

Abstract Wheat (*Triticum aestivum* L.), the basic nutrition for most of the human population, contributes 20% of the daily energy needed because of its, carbohydrate, essential amino acids, minerals, protein, and vitamin content. Wheat varieties that produce high yields and have enhanced nutritional quality will be required to fulfill future demands. Hexaploid wheat has A, B, and D genomes and includes three like but not identical copies of genes that influence important yield and quality. CRISPR/Cas9, which allows multiplex genome editing provides major opportunities in genome editing studies of plants, especially complicated genomes such as wheat. In this overview, we discuss the CRISPR/Cas9 technique, which is credited with bringing about a paradigm shift in genome editing studies. We also provide a summary of recent research utilizing CRISPR/Cas9 to investigate yield, quality, resistance to biotic/abiotic stress, and hybrid seed production. In addition, we provide a synopsis of the laboratory experience-based solution alternatives as well as the potential obstacles for wheat CRISPR studies. Although wheat's extensive genome and complicated polyploid structure previously slowed wheat genetic engineering and breeding progress, effective CRISPR/Cas9 systems are now successfully used to boost wheat development.

Keywords CRISPR/Cas9 · Genome editing · Plant · Transformation · Wheat

Introduction

It is estimated that by 2050, there will be 9.6 billion people in the world (Turhan et al. 2021). To keep the food demand of the booming population, the wheat yield must increase by 50% by 2034 (Zhang et al. 2019). According to a paper published by the World Health Organization (WHO) in 2020, almost 690 million people, 8.9% of the human population, do not have sufficient food to eat. Sustainable food and nutrition security demand sustainable and quality wheat production for the general health and well-being of the masses through responsible consumption and production, which are defined as priority sustainable improvement aims by the United Nations (UN) (Jarvis 2020).

When it comes to grains, wheat (*Triticum aestivum* L.) is one of the most extensively grown and consumed cereal crops in the world. Archaeological archives show that wheat was first grown in the foothills of Karacadag (Diyarbakır, Turkey), known as the Fertile Crescent region, around 10,000–12,000 BC (Karakas et al. 2022). Wheat is the world's most commerced and cultivated product, covering an area of 220 million hectares (mha), with a mean yield of 350 kg. da^{-1} (at 11% moisture content) and a total worldwide production of 773 million tons (USDA 2022). On a continent basis, 45.7% of global wheat production was realized in Asia, 33.5% in Europe, 15.5% in America, 3.3% in Africa, and 2% in Oceania (FAO 2022). More than fifty percent of the world's wheat production is used in human nutrition, and the rest is used in the animal feed and processing industry (Curtis et al. 2002).

Hexaploid bread wheat is thought to be a hybrid of tetraploid wheat (*T. turgidum,* ssp *dicoccum*, AABB,

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2n = 4x = 28) and *Aegilops tauschii* (DD, 2n = 2x = 14) and has a genome size of roughly 17,000 Mbp (Awan et al. 2022; Kou et al. 2023). The wheat genome's this hexaploid structure makes it a valuable model for researching and optimizing genome editing technology (Kim et al. 2018). While people meet of their 19.0% of their calorie needs and 20.8% of their protein needs from bread and other wheat products in their daily diets, a significant part of the daily average nutritional elements required per person is also met from cereals and especially wheat bread (Arslan et al. 2021; Ozturk et al. 2022).

Crop development attempts to improve crop yield, quality, and biotic/abiotic stress tolerance (Erturk et al. 2015; Taspinar et al. 2017). For several decades, crop productivity has been significantly increased using modern agricultural technology (Liu et al. 2021). Today, biotechnologies, breeders, and scientists approach the issue in terms of improving the food quality rather than raising the production of wheat, which is extremely important for human nutrition, in proportion to population growth. Various breeding approaches, such as conventional crossing, molecular marker-assisted, radiation and chemically mediated mutation, and genetic engineering have been efficiently employed to enhance different crop properties (Chaudhary et al. 2019; Singha et al. 2022; Ramesh et al. 2020; Natalini et al. 2021; Puren et al. 2023). However, for polyploid crop breeding the traditional mutagenesis-based breeding procedures are lengthy and arduous (Parry et al. 2009).

Genetic modification is an important research instrument that allows the advance of products with valuable properties and advances the understanding of genes' functions (Lawrenson et al. 2015). Recombination and undirected mutagenesis are both random processes, making it difficult and time-consuming to further improve the current elite germplasm. Contrarily, the high precision of genome editing systems allows for unparalleled control over the mutation technique, permitting multiple advantageous traits to occur rapidly within a single generation (Scheben et al. 2017; Wolter et al. 2019). With technological advances, four nucleases, including meganucleases (MNs), zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) are used for the development and implementation of genome editing systems (Guha et al. 2017; Hajiahmadi et al. 2019; González Castro et al. 2021). TALEN and ZFNs use protein–DNA interactions to identify DNA sequences, but these methods have definite drawbacks, made plasmid construction a challenge, and are also very expensive (Kumar et al. 2015; Soda et al. 2018). TALEN is less costly, easier to construct than ZFN technology, and more specific to the target sequence (Reegan et al. 2016). However, compared to ZFN and TALEN, the CRISPR system is currently considered the most effective, affordable, and flexible cost-effective genome editing technique (Liu et al. 2014; Chang et al. 2016).

The CRISPR/Cas9 system has accelerated the transition to precision breeding by allowing specific genetic manipulation on crops (He et al. 2018; Marwein et al. 2019). When compared to RNA interference (RNAi) and other genome-editing methods, CRISPR/Cas9 has proven more powerful due to its rapidity, heritability, and low off-target mutation rate. The main advantage of this strategy is that the Cas9 transgene may be eliminated in future generations through easy separation or crossing, which offers up a lot of possibilities for applying targeted mutagenesis for speedy crop breeding (Zhang et al. 2016; Zafar et al. 2020).

Another advantage of the CRISPR system over TALENs and ZFNs is the ease with which multiple sites can be targeted simultaneously using multiple sgRNAs (Zarei et al. 2019). Although CRISPR has been used very efficiently for targeted gene knockout in plants, the use of an individual gRNA limits its efficacy and biotechnological applications (Chen et al. 2019a, b). Therefore, recent studies have focused on using multiplexed strategies for multi-locus editing or transcriptional regulation rather than single-guided approaches (Ahmad et al. 2023). CRISPR/Cas9 made it possible to simultaneously modify many quantitative trait loci (QTL) with multiplex genome editing, accelerating the process of improving key crop traits QTL is a specific genetic locus that can result in variations in an organism's phenotypic characteristics, and it controls many yield and quality traits in wheat (Yimam et al. 2021). New research suggests that the range of phenotypic diversity could be increased by CRISPR/Cas9-induced cis-regulatory elements (CREs) mutations in genes affecting productivity traits (Rodríguez-Leal et al. 2017; Wang et al. 2021; Saeed et al. 2022). CRISPR/Cas9 is a valuable tool for knockout non-coding sequences, such as microRNA genes and other non-coding elements (e.g., *cis*-elements of promoters, enhancers, and transposons) in plants (Ding et al. 2016). For this, Cas9 and multiple gRNAs could be used to remove short microRNA fragments (Waheed et al. 2020). Furthermore, CRISPR/Cas9 is a potent device for the activation, transcription, and repression of protein-coding and non-protein-coding genes and can also be said to be an essential tool in plant biology by reversing methylation-induced gene silencing (Liu et al. 2017).

Beginning with a description of the CRISPR/Cas9 molecular platforms, this review concentrates on last advances in genome editing research in wheat. The review aims to assess the application possibilities of CRISPR/Cas9 in wheat. In addition, we list the research of CRISPR/Cas9 in wheat, including the genes, edited, the plasmids employed, the transformation procedure, and the frequency of the resulting changes. Furthermore, it is to bring to the attention of researchers the potential difficulties that may be encountered

in wheat CRISPR studies and the laboratory experience-based solution alternatives for them.

CRISPR/Cas system

History of CRISPR/Cas system

CRISPR is the key element of prokaryotes (such as archaea and bacteria) RNA-mediated adaptive immunity which was first found in *Escherichia coli* in the 1980s and then reported in 1987 by Ishino et al. (1987). At the time, it took many months to sequence these challenging-to-work DNA fragments, and discoverers didn't know where they came from or what role they played in the bacterial cell (Gostimskaya et al. 2022). However, the first surprising experimental information about how the CRISPR system works came when it was discovered that *Streptococcus thermophilus* could become resistant to a bacteriophage by putting a piece of an infectious virus's genome fragment into the CRISPR locus (Doudna and Charpentier 2014; Samso et al. 2015). Due to this discovery, the authors were awarded one of the first patents in the field, and the start of bacterial cultures "vaccination" using CRISPR systems by Danisco in 2005 (Isaacson 2021). In 2012, it was found that the bacterium *Streptococcus pyogenes* (Sp) could be used for gene engineering in eukaryotes. In 2013, researchers reported the first effective use of successful application CRISPR/Cas9 editing for both transient expression and recovery of stable transgenic lines in plants containing *Arabidopsis thaliana, Nicotiana benthamiana, and Sorghum bicolor* (Jiang et al. 2013a, b; Li et al. 2013; Nekrasov et al. 2013).

Biology of CRISPR/Cas system

Many bacteria and most archaea have CRISPR locus and Cas genes, which are part of adaptive immune systems that are guided by RNA and protect against bacteriophage infection and plasmid transfer (Sapranauskas et al. 2011; Wang et al. 2016; Jiang and Doudna 2017). Generally, CRISPR/Cas-mediated systems work in three steps to develop a comprehensive immune response to the intrusive foreign DNA (Gupta et al. 2019). First, at the acquisition or immunization stage, foreign DNA fragments of intrusive plasmids or phages (the protospacers) are introduced into the host CRISPR locus (spacers) between CRISPR RNAs (crRNA) repeat to establish the genetic memory of the cell (Nussenzweig and Marraffini 2020). To cleave foreign RNA or DNA, organisms containing CRISPR obtain DNA fragments from infestive bacteriophages and plasmids, which they then transcribe into crRNAs (Wang et al. 2016). Thus, the host can protect itself from a recurrence of the same invader thanks to the genetic record of the infection (Barrangou et al. 2007; Makarova et al. 2006). Afterward, crRNA is expressed and processed, and Cas proteins are produced. RNA polymerase creates RNA molecules from the CRISPR site's spacer regions; these molecules are known as pre-CRISPR RNAs (pre-crRNAs) (Li et al. 2019a, b; Wang et al. 2022a, b, c). Trans-activating crRNA (tracrRNA) is transcribed upstream of the CRISPR locus and serves two crucial roles: first, stimulating the formation of mature RNase III crRNA, and second, launching crRNA-directed DNA cleavage (Jinek et al. 2012; Nussenzweig and Marraffini 2020). The tracrRNA: crRNA complex then binds to Cas9 to create an active ribonucleoprotein (RNP) complex. In the third phase, crRNA directs Cas proteins to the appropriate target, where they cause a double-strand break (DSB) in the invading foreign genome and thereby shield the host cells from infection (Wang et al. 2022a, b, c). In the event of a second infection, the crRNA spacer hybridizes with a foreign target sequence (protospacer), which in turn activates the sequence-specific cleavage of invasive DNA or RNA by Cas nucleases (Marraffini and Sontheimer 2008; Garneau et al. 2010).

Classification of CRISPR/Cas system

Based on the sequence and structure of the Cas proteins, CRISPR/Cas systems are separated into two classes I (heteromeric multiprotein effector modules) and classes II (single proteins effector modules), 6 types (Class I; type I, III, and IV and Class II; type II, V, and VI), 18 subtypes and multiple variants (Makarova et al. 2020; Ilhan et al. 2021). While there is an enlargement in the classification because of newly investigated systems with recent studies, it is estimated that the current classification is almost complete at the "type" level (Russel et al. 2020). The classification of the CRISPR system is summarized in Table 1.

The Class I systems possess multisubunit crRNA effector complexes and encompass the most common and varied type I and III, which is more common in archaea (nearly all systems) than in bacteria (75%), along with the extremely rare type IV, which consists of primitive CRISPR/Cas loci missing the adaptation module (Makarova et al. 2015; Koonin et al. 2017). Type I includes 8 different Cas operons; type III includes 8 Cas operons and Csm/Cmr complexes and type IV includes 2 Cas operons and 4 DinG/Csf proteins (Koonin et al. 2017). All type I system includes the signature Cas3 protein, which uses the DNase domain and helicase to cleavage the target (Sinkunas et al. 2011). The Cas10 signature gene is found in all Type III systems and codes a multidomain protein, the biggest subunit of Type III crRNA-effector complexes. Type III systems (Type III-A and III-B) are distinguishable because they have distinct genes that code for small subunits, called Csm2 (Type III-A) and Cmr5 (Type III-B) (Makarova et al. 2015). Type IV belongs to a putative class 1 system

Table 1 Classification of different types of CRISPR systems (Loureiro and da Silva 2019; Hillary and Ceasar 2023

	Class 1			Class 2		
	Multiprotein effector modules			Single protein effector modules		
	Type I	Type III	Type IV	Type II	Type V	Type VI
Subtype	A-G	A-F	A-C	A-C	A-I, K, U	A-D
Signature protein	Cas3	Cas10	Csf1	Cas9	Cas12	Cas13 (C2c2)
Target molecule	DNA	DNA/RNA	DNA	DNA	DNA/RNA	RNA
Effectors	Cascade crRNA Cas3	Cmr/Csm crRNA Cas10	–	crRNA tracrRNA Cas9	Cpf1 crRNA	C2c1 crRNA
Pre-CRISPR Processing	Cas6	Cas6	–	RNase III and tracrRNA	Cpf1	–
Nuclease domain	HD	HD and cyclase domain	HD (different type III systems)	RuvC and HNH	RuvC like	2* HEPN
PAM	–	–	–	3', G-rich	5', T-rich	3', A, U or C
Details	Cleaves ssDNA strands	Binds to nascent RNA molecules	Most unknown CRISPR system	Originates blunt DSB	Originates staggered DSB	RNA-guide RNase

and is less recognized as per types I and III. In type IV systems which are generally localized on plasmids, the effector molecular is significantly much smaller (although still composed of multiprotein), and it does not have any domains that are able to cleave targets or insert spacers (cas1 and cas2) (Pinilla-Redondo et al. 2020).

Class II systems are present almost only in bacteria and consist of an RNP complex including a Cas protein and a crRNA (Gasiunas et al. 2012; Scholz et al. 2013; Shmakov et al. 2017). Because of its simplicity and flexibility in practical applications, class II systems have mostly been used to improve CRISPR/Cas-mediated gene editing technologies. Class II is further subdivided into type II, V, and VI systems. Type II and V CRISPR/Cas9 systems contain, respectively, signature Cas9 or Cas12 effectors with DNA interference activity, while type VI systems have Cas13 effectors with RNA interference activity (Zhang et al. 2021a, b, c). In type V systems, effectors such as C2c1, C2c3, and Cpf1 work with a single RuvC nuclease domain, but not the HNH domain (Abudayyeh et al. 2016; Tamulaitis et al. 2017; Perez Rojo et al. 2018).

Type VI depends on the Cas13 protein and two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) nuclease domains (Schindele et al. 2018; O'Connell 2019). Only with Cas13, does collaboration activity after recognition happens, and later, there is non-specific destruction of surrounding transcripts. There is potential for this phenomenon to be developed as an application for RNA targeting because it happens in bacteria but not in animal and plant cells (Gootenberg et al. 2018).

Components of CRISPR/Cas9 system

The type II system based on Cas9 obtained from *Streptococcus pyogenes* (SpCas9) is the most researched and applied among these various CRISPR/Cas systems since its ease of use, adaptability, efficiency, and specificity (Zhang et al. 2021a, b, c). These systems, contain the commonly acknowledged Cas9 effector protein, including RuvC (I, II, and III subdomains) and HNH (His-Asn-His) nuclease domains (Tamulaitis et al. 2017; Janik et al. 2020). Moreover, the Cas9 nuclease comprises two lobes: a nuclease lobe (NUC) and a target recognition lobe (REC). The NUC lobe includes the HNH, the RuvC, and the protospacer adjacent motifs (PAM)-interacting domains. Target DNA strands that are complementary to the short guide RNA (sgRNA) are cut by the HNH nuclease domain, while other target DNA strands are cut by the RuvC nuclease domain (Chen et al. 2019a, b). The recognition site, the REC lobe, recognizes the specific 10–12 nt "seed site" of the sgRNA and has an important role in directing Cas9 to initiate cleavage at the target site (Uniyal et al. 2019).

Cas9 creates an RNP complex with crRNA and a tracrRNA, then this complex cleaves DNA in a crRNA-guided manner (Zhang 2019). sgRNA is another vital element of CRISPR/Cas9. The sgRNA, which is formed when a tracrRNA and a crRNA hybridize, guides the gene-targeting process in CRISPR/Cas (Mali et al. 2013). tracrRNA has three stem-loop hairpin structures that are complementary to the repeat sequence (Chylinski et al. 2013; Nishimasu et al. 2015). The guide sequence in sgRNA is 20 bp long and completely matches the target site's sequence in all genomes. When selecting the target site, care should be taken that the

"NGG" trinucleotide motif, known as PAM, is right next to the protospacer target at its 3' end. The Cas9 cleavage site is found within the protospacer, approximately 3–4 bp upstream of the 5'-terminal end of the PAM (Zarei et al. 2019). Cas9 cannot cleave the target sequence in the lack of PAM (Jiang et al. 2013a, b). CRISPR/Cas9 can be derived from various species, each of which has its unique PAM sequence. For instance, the PAM sequence in *Staphylococcus aureus*, *Campylobacter jejuni, Neisseria meningitidis, Pasteurella pneumotropica,* and *Francisella novicida* are 5'-NNGRRN-3', 5'-NNNNACA-3', 5'-NNNNGATT-3', 5'-GNNNCNNA -3' and 5'-NG -3', respectively (Kim et al. 2017; Fedorova et al. 2020; Hashemi 2020; Caruso et al. 2022; Stevanovic et al. 2022). Also, the sgRNA has a "scaffold" sequence, which is essential for Cas9 to attach to it (Anders et al. 2014).

DNA repair pathways in CRISPR/Cas9 system

When the gRNA and the target sequence are homologous, the HNH and the RuvC catalytic domains of Cas9 cause a DSB in the DNA (Loureiro et al. 2019). DSBs generally be repaired through two major endogenous DNA repair: NHEJ and HDR pathways (Yeh et al. 2019; Zhang et al. 2021a, b, c; Sun et al. 2022). Furthermore, microhomology-mediated end joining (MMEJ) and single-stranded annealing (SSA) are the other repair pathways, both of which are more error-prone (Xue and Greene 2021).

NHEJ is an active, dominant, and error-prone repair mechanism (Chavez et al. 2022). Repairing a DSB with NHEJ typically causes 1–10 bp small insertions or deletions of nucleotides (indels) at the cleavage site (Waters et al. 2014; Ryu et al. 2019). These indels usually reason a frameshift and, eventually, knockouts of the corresponding gene and loss of protein function. Therefore, NHEJ can be used in place of RNAi and other methods of gene silencing (Unniyampurath et al. 2016). HDR differs from NHEJ in that a DNA template (undamaged sister chromatid) including the sequence to be conveyed to the cell alongside Cas9 and the gRNA is necessary (Shrivastav et al. 2008). HDR stimulates specific gene modification or foreign DNA knock-in (precise insertion) when a DNA template is present (Sun et al. 2016).

The occurrence of HDR in nature is low, and eukaryotic cells preferentially use NHEJ instead of HDR through various mechanisms (Pickar-Oliver and Gersbach 2019). One of the reasons for this is since NHEJ is active throughout the cell cycle except in mitosis, while HDR is limited to the S and G2 phases (Ma et al. 2018). Even, in plants, the rate of gene knockouts arising from DSB repair via the NHEJ mechanism ranges from 30 to 70% and can even reach 100% in some circumstances (Rozov et al. 2019). Accordingly, many scientists have tried strategies to develop the HDR repair mechanism process and/or suppress the NHEJ pathway by targeting essential components for more effective HDR-mediated precise genome editing (Riesenberg and Maricic 2018; Jayavaradhan et al. 2019; Li et al. 2019a, b).

CRISPR/Cas9 geneediting system in wheat

Today, functional genomics and molecular breeding studies in wheat have gained momentum as the CRISPR/Cas9 technique is used to target more than one homoallele simultaneously (Li et al. 2021a, b). Forward genetic methods like mapping-based cloning of quantitative trait loci (QTLs) and insertion mutagenesis (T-DNA or transposon tag) have helped identified many genes and alleles (Appels et al. 2018; Bettgenhaeuser and Krattinger 2019; Pereira et al. 2020; Sahu et al. 2020). These genes have been used to improve wheat yield with reverse genetic methods such as CRISPR/Cas9, ZFN, TALENs, meganucleases, RNAi, overexpression, and target-induced local lesions in the genome (TILLING) (Aglawe et al. 2018; Savadi et al. 2018; Nerkar et al. 2022). The development of gene editing technologies and understanding of gene functions in wheat have significantly increased studies on modifications in genes that regulate crucial traits such as yield, quality, biotic/abiotic stress tolerance, and hybrid seed production (Lv et al. 2020; Usman et al. 2021; Liu et al. 2022). The investigations on the CRISPR/Cas9 methodology in wheat are summarized in Table 2, which also includes information on the cultivar or genotype, targeted gene(s), gene function, genome editing approach, CRISPR vector, sgRNA promoter, and delivery techniques.

Improving wheat yields

An enormous rise in wheat production is needed to feed the rising human population. Wheat's global genetic improvements of less than 1% per year are woefully inadequate to meet the expected future demand (Ober et al. 2021)). (Kumar et al. 2019)). This fact has prompted researchers to explore and breed new types of wheat. In late years, scientists have used the CRISPR/Cas9 technique for ameliorating wheat production and quality, which is a much faster and more precise manner to create more allelic variations and silence several regulatory genes (Zhang et al. 2021a, b, c)). Achieving high yields to increase production has been the top priority in wheat breeding. Specific characteristics linked to grain yield include tiller number per panicle, grain number and size per panicle, grain weight, and grain size (Liu et al. 2022)). Gupta et al. (2022) modified the microRNA-156 recognition elements (MRE) of TaSPL13 via CRISPR/Cas9 in hexaploid wheat and observed increased grain size and number in TaSPL13 mutants. The yield of bread wheat is highly dependent on the architecture of its inflorescence, which determines the number of grains per spike (Gao et al. 2019).

Table 2 List of CRISPR/Cas9-based genome editing system applications in wheat

Cultivar or genotype	Targeted gene(s)	Gene fnction	Genome editing method	CRISPR vector	sgRNA promoter	Delivery method	References
Wheat yields							
Triticum aestivum cv. Fielder	*TaSPL13*	Improves grain yield and other agronomic traits	CRISPR/Cas9	–	*TaU6*	*Agrobacterium* (strain GV3101)	Gupta et al. (2022)
Triticum aestivum cv. Fielder	*DUO-B1*	Regulating spike inflorescence architecture	CRISPR/Cas9	pBUE411	–	*Agrobacterium*	Wang et al. (2022a, b, c)
Triticum aestivum cv. Kenong 199	*FT-D1*	Flowering time	CRISPR/Cas9	pBUE414	*PIN2*	Biolistic	Chen et al. (2022a, b)
Triticum aestivum cv. Bob-white	*TaGW7*	Affects grain shape and weight	CRISPR/Cas9	pA9Cas9sg	–	Protoplasts transformation	Wang et al. (2019a, b)
Triticum aestivum cv. Kenong 199 and Bobwhite	*TaLOX2*	Affects grain development and storability	CRISPR/Cas9	pGE-sgRNA	*TaU6*	Particle bombardment	Zhang et al. (2016)
	TaGASR7	Implicates in the control of grain length and weight					
	TaGW2	Genetic determinant of grain weight					Zhang et al. (2018)
Triticum aestivum cv. Fielder	*TaQsd1*	Control seed dormancy	CRISPR/Cas9	PubiMMCas9	*OsU6*	*Agrobacterium* (strain EHA101)	Abe et al. (2019)
Triticum aestivum cv. Fielder	*TaCKX2*	Regulating wheat spike mor-phogenesis	CRISPR/Cas9	pMJ806	*TaU6*	*Agrobacterium* (strain EHA105)	Zhang et al. (2019)
Grain quality							
Triticum aestivum cv Pakistan 2013 and Borlaug 2016	*TaIPK1*	Phytic acid biosynthesis	CRISPR/Cas9	pENTR4	*TaU6*	*Agrobacterium* (strain LBA4404)	Ibrahim et al. (2022)
Triticum aestivum cv BW208, THA53, and durum wheat cv DP	*α-gliadin*	Components of gluten complex	CRISPR/Cas9	pANIC-6E	TaU6	Particle bombardment	Sánchez-León et al. (2018)
Triticum aestivum cv. Fielder	*γ-gliadin*	Components of gluten complex	CRISPR/Cas9	pBUE411	–	*Agrobacterium* (strain EHA105)	Liu et al. (2023)
Wheat line CB037B	*TaPDI*	Formation of glutenin macropolymers	CRISPR/Cas9	pWMB110	*Ubi*	*Agrobacterium* (strain C58C1)	Hu et al. (2022)
Triticum aestivum cv. Zheng-mai 7698 and Bobwhite	*TaSBEIIa*	Effects amylose and starch content	CRISPR/Cas9	pCXUN-Cas9	*TaU6*	Particle bombardment	Li et al. (2020)
Triticum aestivum cv. Fielder	*Pinb*	Formation of soft wheat	CRISPR/Cas9	–	–	*Agrobacterium*	Zhang et al. (2021a, b, c)
	Waxy	Amylose synthesis					
	Ppo	Formation of the colour of wheat products					
	Psy	Carotenoid Biosynthesis					
Triticum aestivum cv. Zheng-mai 7698	*TaARE1*	Regulates nitrogen use effi-ciency and grain yield	CRISPR/Cas9	pC1390-YFP	*TaU6*	Particle bombardment	Zhang et al. (2021a, b, c)
Triticum aestivum cv. Cadenza	*TaASN2*	Asparagine synthetase	CRISPR/Cas9	pRRes209.481	–	Particle bombardment	Raffan et al. (2021)
Triticum aestivum Nicotiana benthamiana	*TaPDS*	Encodes enzymes involved in the carotenoid biosynthetic pathway	CRISPR/Cas9	pBI121	*CaMVE35S*	*Agrobacterium* (strain GV3101)	Upadhyay et al. (2021)
	TaINOX	Cellulose production					

Table 2 (continued)

Cultivar or genotype	Targeted gene(s)	Gene fnction	Genome editing method	CRISPR vector	sgRNA promoter	Delivery method	References
Biotic stress							
Triticum aestivum cv. Fielder	*TaCIPK14*	Defense responses against pathogens	CRISPR/Cas9	VK005-6	*TaU6*	*Agrobacterium*	He et al. (2022)
Triticum aestivum cv. KN199, Xinong511 (XN511), Shi4185 (S4185) and Xiaoyan60 (XY60)	*TaMLOX*	Provides resistance to powdery mildew	CRISPR/Cas9	pJIT163	*Ubi*	Particle bombardment	Li et al. (2022)
Triticum aestivum cv. KN199	*TaEDR1*	Plays a negative role in the defense response against powdery mildew	CRISPR/Cas9	pJIT163	*Ubi*	Particle bombardment	Zhang et al. (2017)
Triticum aestivum cv. Fielder	*TaNFXL1*	Develops resistance to Fusarium head blight	CRISPR/Cas9	pVB29	–	–	Brauer et al. (2020)
Triticum aestivum cv. Cadenza	*TaBAK1-2*	Regulator of plant immunity and development	CRISPR/Cas9	pUC19	*OsU6*	Particle bombardment	Hahn et al. (2021)
Triticum aestivum cv. Cezanne, Goncourt, and Prevert	*Ta-eIF4E* *Ta-eIF(iso)4E*	Susceptibility (S) factors		pFH11 pFH12	*OsU3*		
Abotic stress							
Triticum aestivum cv. Giza168	*TaSal1*	Improve drought stress tolerance	CRISPR/Cas9	pCas2143	*PvUbi1*	Biolistic	Abdallah et al. (2022)
Triticum aestivum cv Fielder	*TaHAG1*	Regulator to strengthen salt tolerance	CRISPR/Cas9	pBUE411	*Ubi*	*Agrobacterium*	Zheng et al. (2021)
Triticum aestivum cv Chinese	*TaDREB2, TaERF3*	Abiotic stress-responsive transcription factor	CRISPR/Cas9	pJIT163-2NLSCas9	*TaU6*	Protoplast transformation	Kim et al. (2018)
Triticum aestivum cv CB037	*TaMBF1c*	Upregulated in response to heat stress	CRISPR/Cas9	pBUE411	–	*Agrobacterium* (strain EHA105)	Tian et al. (2022)
Hybrid seed production							
Triticum aestivum cv CB037	*TaNP1*	Regulate tapetum degeneration and pollen exine formation	CRISPR/Cas9	pOPGR	*TaU3p,TaU6p, OsU3p,OsU6p*	Particle bombardment	Li et al. (2020)
Triticum aestivum cv Fielder and SBC0456D	*Ms45*	Male fertility	CRISPR/Cas9	–	*TaU6*	*Agrobacterium*	Singh et al. (2018)
Triticum aestivum cv. Fielder and Gladius	*Ms1*	Male fertility	CRISPR/Cas9	pUC57	*TaU6*	*Agrobacterium* (strain AGL1)	Okada et al. (2019)
Triticum aestivum cv. Fielder	*TaCENH3*	Induces paternal haploid induction	CRISPR/Cas9	–	*TaU6*	Biolistic	Lv et al. (2020)
)*Triticum aestivum* L	*TaPLA*	Triggers haploid induction	CRISPR/Cas9	–	–	*Agrobacterium*	Liu et al. (2020a, b)
Triticum aestivum cv. Fielder and Ningchun4 Transgenic wheat line H29	*TaWaxy, TaMTL*	Haploid induction	CRISPR/Cas9	pWMB110	*TaU3*	*Agrobacterium* (strain C58C1)	Liu et al. (2020a, b)

Wang et al. (2022a, b, c) described DUO-B1 as a transcription factor encoding an APETALA2/ethylene response factor (AP2/ERF) that modulates spike blooming architecture in wheat. Gene editing in DUO-B1 created using CRISPR/Cas9 resulted in mild supernumerary spikelets, a rise in grain number per spike, and a considerable yield under field circumstances, without affecting other essential agronomical characteristics. It is known that DUO-B1 is not used in plant breeding, and it is predicted that wheat yield can be increased further with CRISPR/Cas9 studies to be carried out. A mutation in FT-D1 (flowering time gene) led to a rise in total spikelet number and heading date when compared to the wild type. In addition, one simple, easy-to-use, and strong marker designed corresponding to the polymorphic location of FTD1 showed that this specific G indel had been favorably selected for adaptation to varying environmental conditions (Chen et al. 2022a, b). By modifying *TaGW7* homologs in the wheat B and D genomes using the CRISPR/Cas9 system, researchers were able to improve both grain size and weight (Wang et al. 2019a, b). The growth of the endosperm and the filling of the grain are the primary factors that determine the grain yield and nutritional quality. GRAIN WIDTH AND WEIGHT2 (*GW2*) gene encoding by the RING-type E3 ubiquitin ligase is arising as a major genetic driver of grain weight in cereal (Achary and Reddy 2021). To be silenced the TaGW2 lipoxygenase gene using CRISPR/Cas9 increased grain weight as well as protein content in wheat (Zhang et al. 2018). The CRISPR/Cas9 technology was applied to knockout the *TaLOX2* gene in wheat, which changed grain size and weight and improved wheat's ability to be stored (Zhang et al. 2016). Regardless of cultivar background, thousand-grain weight was dramatically raised when CRISPR/Cas9 was used to simultaneously target all three TaGASR7 homeolog (Zhang et al. 2016).

Seed dormancy and germination are the most significant agronomic and trading feature of cereal crops (Cheng et al. 2022). The most important among dormancy genes is the quantitative trait locus for seed dormancy 1 (Qsd1), which was identified in barley. Abe et al. (2019) performed mutations in *TaQsd1* genes in subgenomes A, B, and D with CRISPR/Cas9 in wheat, and the mutant plants indicated an importantly longer seed dormancy period than the wild type. Knockout of the *TaCKX2-D1* (cytokinin oxidase/dehydrogenase) gene with the CRISPR/Cas9 in wheat increased grain yield (Zhang et al. 2019).

Improvement of wheat grain quality

Agriculture needs to meet increasing food demands by bio-enriching high nutritional value crops through several food technological procedures to struggle with malnourishment and feed the growing global population (Rao and Wang 2021). Therefore, CRISPR/Cas offers a significant amount of promise for ensuring food security and feeding the expanding population of the globe through environmentally responsible agricultural practices. CRISPR/Cas9 for quality traits amelioration requires some specific characteristics; physical characteristics (e.g., size, color, texture, and fragrance), nutrients (e.g., lipids, minerals, proteins, and starch), and bioactive compounds (e.g., carotenoids, flavonoids, lycopene, and γ-aminobutyric acid) (Liu et al. 2021; Achary and Reddy 2021; Vats et al. 2022). Phytic acid (PA) has a high density of negative charges owing to its six phosphate groups, which are partly ionized at physiological pH and balanced by cations, mainly sodium ions (Nassar et al. 2021). Therefore, it can form insoluble complexes with minerals, proteins, enzymes, and starches (Sarkhel and Roy 2022). In a plant-based diet, PA is thought to be the most potent inhibitor of the bioavailability of Fe and Zn (Kumar et al. 2017a, b). Zn deficiency, called "hidden hunger," is a widespread public issue worldwide and brings many health problems (Waqeel and Khan 2022). Additionally, recent research has used CRISPR/Cas9 to target-related genes in the inositol pathway to develop the bioavailability of zinc. Ibrahim et al. (2022) created mutations on three *TaIPK1* homologs, *TaIPK1.A*, *TaIPK1.B*, and *TaIPK1.D*, with the CRISPR/Cas9 technique in wheat. An examination of the sequences of T0 plants revealed the deletion of 1–23 nt and even an addition of 1 nt in different lines. Conventional breeding methods cannot lower gluten concentration in bread wheat due to 100 genes and pseudogenes placed in tandem at the Gli-2 loci of 6A, -B, and -D chromosomes for α-gliadins (Jouanin et al. 2019). Using the CRISPR/Cas9 technique to target a conserved section of the α-gliadin genes, the researchers designed two sgRNAs and obtained wheat lines with low-gluten (Sánchez-León et al. 2018). In addition, low-gluten wheat mutants have been developed by knockout of the γ-gliadin genes (*Gli-γ1* and *Gli-γ2*) (Liu et al. 2023). Hu et al. (2022) used CRISPR/Cas9 to target wheat's protein disulfide isomerase (*PDI*) gene. As a result, seven homozygous *TaPDI* mutants were obtained, and it was reported that the mutants significantly reduced glutenin macro polymers, especially PDI-abd-6. Regulation of this gene may be suggested to improve the glutenin macro polymers accumulation and processing features of wheat dough.

Resistant starch (RS) and amylose-rich foods have considerable potential to benefit human health and decrease the risk of serious noninfectious sicknesses (Kim et al. 2021). Li et al. (2020) induced mutations in the *TaSBEIIa* gene in two different wheat varieties, Zhengmai 7698 (winter) and Bobwhite (spring–summer), with the CRISPR/Cas9 technique, and wheat with high amylose content was produced. Wheat with improved grain quality such as dough color, grain hardness, and starch quality has been developed by knockout of the *Pinb, Waxy, Ppo*, and *Psy* genes using the CRISPR/Cas9 technique by Zhang et al. (2021a, b, c).

Nitrogen (N) as fertilizer has been used widely in agriculture to develop wheat yield to meet the increasing demands for food needs (Anas et al. 2020). Unfortunately, current wheat cultivars have a low N-use effectiveness (NUE), therefore the overuse of nitrogen fertilizer in agriculture contributes to ecological degradation and environmental pollution. For this purpose, Zhang et al. (2021a, b, c) extracted and identified three *TaARE1* (abnormal cytokinin response1 repressor1) homeologs from wheat cv. ZhengMai 7698. The authors next used CRISPR/Cas9-mediated targeted mutagenesis to generate a panel of transgene-free mutant lines with either double- or triple-null taare1 alleles. These lines included greater tolerance to N absence, delayed senility, and improved grain production under field conditions. *ARE1* orthologs can be used to grow high-yielding wheat that can also be expanded to other important grain crops. Free asparagine is the precursor for acrylamide, which is produced when meals made from wheat are baked, toasted, or subjected to other high-temperature processes (Baskar et al. 2018). The authors knocked out the asparagine synthetase gene (*TaASN2*) in wheat using the CRISPR/Cas9 technique, revealing that the content of free asparagine in all 6 *TaASN2* alleles is diminished compared to wild species (Raffan et al. 2021). Upadhyay et al. (2013) targeted *TaINOX* and *TaPDS* genes, which encode enzymes involved in the cellulose production and carotenoid biosynthetic pathway respectively, with CRISPR/Cas9 technology using one expression construct in wheat. In addition to obtaining a sequence with a 24 bp deletion in the *TaINOX* gene among the editing spectra, the authors found that the mutagenesis efficiency was 18–22%.

Enhancement resistance to diseases caused by biotic stresses in wheat

Although the high diversity of wheat in the world, there has not been an important rise in yield and production in late years because of biotic/abiotic stress conditions (Arslan et al. 2021). Various organisms including bacteria, fungi, viruses, and insects are the factors of biotic stresses (Maharajan et al. 2022). Wheat is susceptible to infection from a wide variety of pathogens, which can result in several diseases (Wulff and Krattinger 2022). Diseases like these can be managed with the use of a variety of fungicides, herbicides, and insecticides, but these chemicals pose serious threats to both humans and animals, as well as the environment (Aydin et al 2021; Tănăsescu and Lite 2022). Therefore, a better way CRISPR/Cas9 technology can target genes sensitive to biotic stress and induce plant resistance.

One of the most damaging wheat diseases, wheat stripe rust induced by the *Puccinia striiformis f. sp. tritici* (Pst) causes major losses in wheat output around the world (Esmail et al. 2023). Calcineurin B-like protein (CBL)-interacting protein kinases (CIPKs) has been shown to be related to resistance throughout plant-pathogen interactions (Xiao et al. 2022). Research on wheat has shown that the knockdown of the *TaCIPK14* gene can lead to wheat developing resistance to Pst. Conversely, overexpression of *TaCIPK14* increased whea's sensitivity to Pst by reducing the defense response by suppressing reactive oxygen species (ROS) accumulation and pathogenesis-relative genes (He et al. 2022). The MILDEW LOCUS O (MLO) gene was first recognized in barley and later emerged as a susceptibility (*S*) gene in mono-dicotyledons (Borrelli et al. 2018; Langner et al. 2018). Degrement of function mutations on *MLO* genes leads to resistance to powdery mildew in both monocot and dicot plants (Shan et al. 2013; Wang et al. 2014; Gil-Humanes et al. 2017; Li et al. 2022). Modification of the *TaMLOX* gene using CRISPR/Cas9 provided resistance to powdery mildew in four elite wheat cultivars without loss of crop growth and yield (Li et al. 2022). CRISPR/Cas9 technique-mediated knockout of improving disease resistance1 (*TaEDR1*) showed enhanced resistance in hexaploid wheat to powdery mildew caused (*Blumeria graminis)* (Zhang et al. 2017). Fusarium head blight (*Fusarium graminearum)* is another devastating disease of wheat (Xu et al. 2022). A mycotoxin virulence factor called deoxynivalenol (DON) encourages the growth of the *Fusarium graminearum* in the floral tissues of wheat (McLaughlin et al. 2021). Brauer et al. (2020) showed DON-induced transcriptional alterations in wheat spikelets to reveal the effects of DON exposure on plant cell function. For this purpose, they targeted the *TaNFXL1* gene using the CRISPR/Cas9 method and showed that this gene increased fusarium head blight resistance in wheat.

In addition to fungal diseases, virus infections are also fundamental problems in wheat farming. That's why scientists have started using CRISPR/Cas9 to crop tolerance to viruses. The genomes of viruses evolve rapidly and may include double-stranded DNA/RNA (dsDNA/dsRNA) and single-stranded DNA/RNA (ssDNA/ssRNA) (Nasir and Caetano-Anollés 2015). Both *Ta-eIF4E* and *Ta-eIF(iso)4E* genes encode translation initiation factors that are well conserved. They also act as S factors necessary for the life cycle of plant viruses, which are members of the Potyviridae family. Bread wheat mutant's knockout of *Ta-eIF4E* and *Ta-eIF(iso)4E* by CRISPR/Cas9 technique is susceptible to wheat spindle streak mosaic virus (WSSMV) and wheat yellow mosaic virus (WYMV) is predicted to be resistant (Hahn et al. 2021).

Enhancement of abiotic stress tolerance in wheat

Among the most common abiotic stresses plants face are those brought on by drought, salinity, temperature extremes, lack of nutrients, and heavy metals (Taspinar et al. 2018). In plants under abiotic stresses, changes such as Ca^{2+}

concentrations, ROS, and abscisic acid (ABA) activation occur (Lee et al. 2022; Waseem et al. 2022). Several studies have shown that abiotic stressors have a significant negative impact on the growth and production of grains. In some cases, abiotic stress factors can reduce a plant's output by more than 50% (Yigider et al. 2021). Many recent studies have reported that specific target genes such as *S* and tolerance (*T*) genes are used to improve wheat tolerance to abiotic stress by CRISPR/Cas9 application. By targeting these genes, activation of specific signal transduction pathways and transcriptional rearrangement that can activate protecting mechanisms (i.e., cell detoxification, osmotic regulation, modulation of stress signaling, and improved stress-induced damage) can be induced.

Regarding wheat's ability to tolerate drought, Abdallah et al. (2022) used multiplex CRISPR/Cas9 techniques to edit the *TaSal1* gene; the results showed that five *Sal1* mutant seedlings grew better under drought stress than the wild-type control. Polyploidy is common in plants and has a vital role in both the process of plant evolution and the differentiation of species (Sabooni et al. 2022). Since polyploids are known to be more adaptable to environmental stresses against diploid plants, it is thought that they can develop new properties of diploids through gene editing studies. Zheng et al. (2021) mutated the *TaHAG1,* an important regulator of salt tolerance in wheat using CRISPR/Cas9. Mutations on the *TaHAG1* gene increase salt tolerance by controlling ROS production and signal specificity. Kim et al. (2018) published their findings that two genes (*TaDREB2* and *TaERF3*) related to stress were edited via the CRISPR/Cas9 in wheat protoplasts. Consequently, demonstrated the ability to generate new stress-resistant variants in plant strains expressing CRISPR/Cas9 with this modification. Wheat harvests are being negatively impacted by climate change because the plant's reproductive system is being damaged by the increased temperatures (Ullah et al. 2022). Therefore, heat stress significantly reduces global wheat output and results in significant yield losses worldwide (Wu et al. 2022). When wheat is subject to high temperatures, the *TaMBF1c* gene is activated. Tian et al. (2022) employed the CRISPR/Cas9 technique to understand the role of Multiprotein Binding Factor 1 (MBF1) in heat stress tolerance in wheat. The finding display that TaMBF1c is evolutionarily conserved in di-, tetra-, and hexaploid wheat species, and knockdown-out mutants are heat sensitive.

Speeding wheat hybrid seed production

As a result of heterosis, hybrid crop types frequently exhibit superior features in comparison to their homozygous parents (Schnable and Springer 2013). Wheat hybrids have up to 20% yield advantages compared to elite wheat varieties and are more resistant to environmental stress factors (Mühleisen et al. 2014). The creation of male-sterile and double-haploid wheat plants can significantly accelerate the process of creating hybrid seeds. Wheat's high genetic redundancy because of the allohexaploid makes it challenging to create nuclear recessive male sterile mutants through spontaneous mutation, chemical or physical mutagenesis technique. Therefore, CRISPR/Cas9 systems have also been utilized to develop other crop qualities such as fertility leading to the improvement of new plant species and haploid lines (Liu et al. 2022). Li et al. (2020) have investigated the editing of *TaNP1* pollen fertility genes using CRISPR/Cas9 systems. In the study, various RNA polymerase III promoters, namely TaU3p-U6p and OsU3p-U6p, were used and the TaU3 promoter was determined as the most effective. Editing three homologous alleles encoding the *TaNP1* gene resulted in completely male-sterile mutants in wheat. The male sterile 45 (*Ms45*) gene homolog in wheat was knockout out using the CRISPR/Cas9 technique, and eventually, male sterile plant mutants were produced (Singh et al. 2018). Recently, completely male sterile mutants have been obtained by creating frameshift mutations in the *MS1* gene in wheat via the CRISPR/Cas9 technique (Okada et al. 2019). Editing the CENTROMERIC HISTONE (*CENH3*) gene by genetic engineering induces paternal haploid induction (HI) (Wang et al. 2019a, b). Lv et al. (2020) use the CRISPR/Cas9 technology to create a commercially viable paternal HI line in wheat with a 7% HI ratio and TaCENH3 gene alterations. The *TaPLA* gene was knockout using the CRISPR/Cas9 technique in the A and D genomes of wheat, resulting in an HI ratio of 2–3% (Liu et al. 2020a, b). The *TaWaxy* and *TaMTL* genes in wheat were genome editing with CRISPR/Cas9 for haploid plant production, and the highest mutation frequency of 80.5% was obtained using the TaU3 promoter (Liu et al. 2020a, b).

Potential obstacles and the laboratory experience-based solution alternatives for wheat CRISPR studies

Wheat has much less genome editing research than other plant species because critical components of the CRISPR system, such as the Cas9 enzyme, are still being developed to fit the properties of the wheat genome (Dayani et al. 2019). Recently, the CRISPR/Cas9 system has been successfully used to increase the characteristics of agricultural crops, but there are still some problems with the CRISPR/Cas9 editing technology that is currently available (Fig. 1). These problems include off-target impacts, the scope of editing, and problems with plant genetic transformation methods.

The targeting specificity of CRISPR/Cas9 is still an ongoing debate in research. Cas9's ability to cleave genomic regions that are outside of its intended target results in off-target that are not intended (Liu et al. 2022). The targeting

accuracy of Cas9 endonuclease is known to be heavily influenced by PAM (3–5 bp) sequences positioned at the 3' end of the target region of the genome and the 20 nt sgRNA (Manghwar et al. 2020). Even though there are mismatches in the target sequence, the system can still work normally (Mahfouz et al. 2014). The presence of off targeting can cause chromosomal rearrangements that may lead to damage to functional gene activity, resulting in damage to incorrectly matched genomic loci (Manghwar et al. 2020). Therefore, it should be recognized that there will always be some risk of off-target mutagenesis in CRISPR/Cas9 studies and new strategies should be considered to minimize this possibility. There are essentially two approaches to solving this problem. The first strategy involves improving specificity to decrease the likelihood of off-target impacts, the second strategy requires experimenting to minimize the effects of false results, reducing the potential for off-target mutagenesis.

Correctly designing the sgRNA is a vital stage in maximizing the effectiveness of the CRISPR/Cas9 technique. Many biotech companies are developing software for sgRNA design, and CRISPy-web, CRISPR-P, CRISPR-ERA, Cas Designer WheatCRISPR, and CHOPCHOP are some of them (Lei et al. 2014; Liu et al. 2015; Park et al. 2015; Blin et al. 2016; Labun et al. 2016; Cram et al. 2019). Badhan et al. (2021) two sets of sgRNA were designed using the CHOPCHOP tool. Off-target effects can only be predicted with accurate information about the sequence of the genome. The wheat genome is complicated because of its allohexaploid structure or containment of different genomes (AABBDD) (Dayani et al. 2019). sgRNAs designed for wheat genome editing studies can bind to off-target regions in other subgenomes in addition to the targeted region. Therefore, it is required to design sgRNA from conserved regions in all genomes of wheat and to identify non-target regions. For this, various software is available that predict whether the sgRNA genome is the target of the CRISPR/Cas9 and, if possible, the off-target regions in the genome to which it can possibly bind. The sgRNAs can be evaluated using tools such as CasOFFinder, CasOT, CCTop, and CRISPR Multitargeter (Bae et al. 2014; Xiao et al. 2014; Prykhozhij et al. 2015; Stemmer et al. 2015). Lee et al. (2019) employed the CasOFFinder tool, which could identify probable off-target regions throughout the genome, to assist decrease the off-target proportion. Cas9 enzyme cuts three nucleotides upstream of the PAM site in the CRISPR/Cas9 mechanism. The cutting effectiveness of Cas9 varies in some cases with the specialty of the PAM sequences, so selecting the correct PAM sequence can decrease the rate of off-target mutations (Yuan et al. 2017; Zhu 2022). Scientists have shown that by mutating some regions of the enzyme, they can limit Cas9 off-target impacts (Kleinstiver et al. 2016; Liu et al. 2022). This restriction was made easy with the development and application of the engineered SpCas9

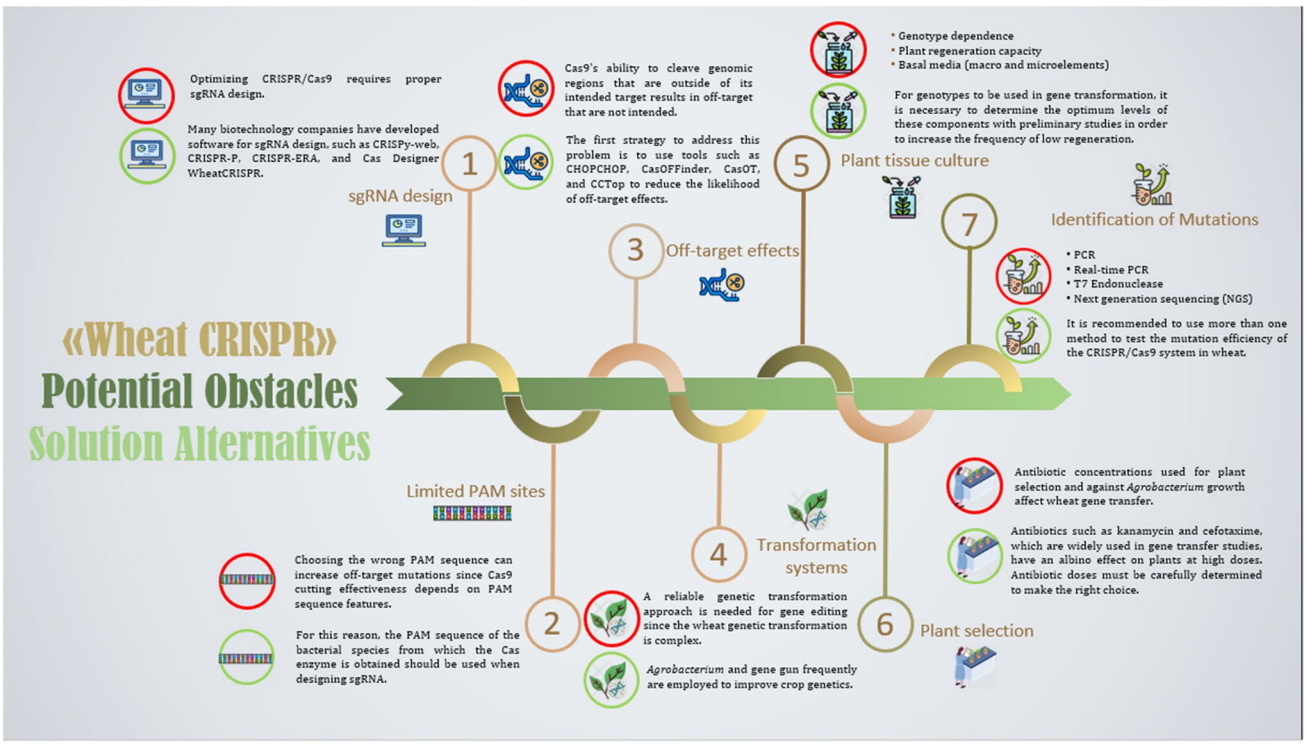

Fig. 1 Possible obstacle in wheat CRISPR/Cas9 studies and lab-based solution alternatives

variant SpRY, which can target DNA PAM-free in plant cells (Ren et al. 2021).

Although CRISPR technology gives several opportunities to modify the wheat genome, the fact that wheat is a monocotyledonous plant makes vector transfer to cells and the regeneration of transgene cells more challenging. The plant transformation process is the transfer of foreign genetic material into plant cells, tissues, or organs to change the plant's trait(s) or phenotype (Chanyalew et al. 2019). When compared to dicotyledons, monocotyledons have fewer explant sources from which somatic embryos can be formed. Dicotyledonous plants frequently use hypocotyls and leaf fragments for transformation, while monocotyledonous plants use seed embryos and scutellum (Hiei et al. 1997). Also, an important rise in callus formation has been suggested after mechanical injury to tissue surfaces (Aydin et al. 2011).

An effective and stable genetic transformation mechanism is required for gene editing of agricultural crops with the CRISPR/Cas9 technique. For crop genetic enhancement, frequently used methods include *Agrobacterium* and the gene gun (microprojectile or particle) technique (Kapusi and Stoger 2022; Wijerathna-Yapa et al. 2022). Most wheat CRISPR/Cas9 investigations have used biolistic gene transfer since the genetic transformation is difficult (Zhang et al. 2018). Biolistic is a DNA transformation technique in which metal particles coated with foreign genes (multiple genes, RNA sequences, or proteins) are blasted with high-pressure gas and incorporated into recipient cells (Twyman and Christou 2004; Liu et al. 2019; Zhi et al. 2022). The first effective genetic transformation of bread wheat using biolistic was performed by researchers at the University of Florida (USA) (Vasil et al. 1992). The application of the biolistic technique makes it possible to add very large DNA fragments to wheat. According to Partier et al. (2017), a 53-kilobyte linear cassette was successfully inserted into the bread wheat genome through the biolistic transformation technique. It has proven to be more effective than *Agrobacterium*-mediated techniques while less dependent on the specific genotype used (Wijerathna-Yapa et al. 2022). However, the efficacy of biolistic approaches is very limited because of constraints in current tissue culture regeneration systems and multiple-copy insertions, and their transformation capacity is low (Sparks and Jones 2004; Liu et al. 2022). Although other techniques for crop transformation have been tried and tested (such as polyethylene glycol, liposomes, silicon carbide, and microinjections), their drawbacks prevent their widespread application. For example, these methods are time-consuming and costly, and they rely on complex infrastructures (tissue culture regeneration systems) and specific genotypes (plants) (Que et al. 2014).

Agrobacterium-mediated techniques, on the other hand, often show a higher frequency of single-site insertions (Dai et al. 2001). *Agrobacterium* (gram-negative soil bacterium) is successfully and widely used for plant genetic transformation. *Agrobacterium* strains have plasmids called Ti plasmid (tumor-inducing) (Aglawe 2018). *Agrobacterium* is inherently capable of infecting injured dicotyledonous plants, and then bacterial T-DNA is integrated into the host genome and expressed owing to the host DNA repair mechanism (Hiei et al. 1997). Additionally, the Monsanto researchers were the first to paper on the generation of transgenic wheat via the *Agrobacterium*-dependent transformation method (Cheng et al. 1997).

Each genetic transformation process includes three important steps: susceptibility of infected tissue to *Agrobacterium,* the regeneration capacity of the target tissue, and an effective selection system for the recovery of transformed plants (Wijerathna-Yapa et al. 2022). Although genome editing represents an unprecedented technological advance, its use for agricultural enhancement is hampered by low plant regeneration frequencies and genotype dependency. The efficiency of *Agrobacterium*-mediated gene transformation is affected by a variable of factors. Different genetic backgrounds, as well as physical and metabolic factors, cause various results in *Agrobacterium* infection (Das et al. 2002). Genotype dependence is the most well-known obstacle among variants of the same plant species. 'Fielder' and 'Bobwhite' are two examples of wheat varieties bred with the purpose of working in wheat gene transformation experiments (Ishida et al. 2015). Especially, the Bobwhite SH98-26 variety shows a TF of over 70% in gene transfer with the biolistic method (Pellegrineschi et al. 2002; Ye et al. 2022). *The Agrobacterium*-mediated transfer method is not appropriate for all agricultural crops and tissues, as it depends on the plant tissue culture regeneration system (Liu et al. 2022). The transformation frequency (TF) of every plant, including wheat, is significantly influenced by the conditions under which it was grown in tissue culture. Several significant factors in tissue culture that impact TF in various species or plant organs are known to be associated with the basal media, including macro and microelements and vitamins (Hesami et al. 2021). Multiple reports on wheat transformation have demonstrated that MS (Murashige and Skoog 1962) or a modified MS-based medium is appropriate for inoculation and co-cultivation (Ding et al. 2009; Alikina et al. 2016; Hayta et al. 2021). Greer et al. (2009) enhanced the biolistic transformation efficiency of elite wheat (cv. Superb) calli sevenfold by adding 62.56 mM ammonium nitrate to the callus production medium. However, there are also some studies that show that high amounts of salts in the environment during transformation reduce the effectiveness of *Agrobacterium*. Therefore, reducing MS salts to 1/10 at the transformation stage is recommended (Carvalho et al. 2004; Wang et al. 2022a, b, c).

The utilization of genes involved in regeneration throughout in vitro culture can increase plant transformation's efficiency. The authors indicated that overexpression of the wheat gene *TaWOX5* importantly rises immature embryo transformation efficiency while being less genotype dependent (Wang et al. 2022a, b, c). Overexpression of the *TaCB1* gene, from the same gene family, intensely enhanced the transformation efficiency of numerous wheat cultivars without genotype dependence (Ke et al. 2021). Furthermore, two studies stated that the overexpression of *TaCB1* and *TaWOX5* in wheat callus did not inhibit both shoot differentiation and root development. The findings of Qiu et al. (2022) showed that the modulation of *TaGRF4* and *TaGIF1* genes in wheat led to an increase in both the frequency of genome editing and the rate of regeneration. Raman et al. (2022) described a method for delivering proteins that reduce plant defense and/or boost transformation using *A. tumefaciens* that has been engineered with a T3SS. In practice, T-DNA was co-transfected with *P. syringae* effectors such AvrPto, AvrPtoB, or HopAO1, which have been indicated to increase transformation efficiency in Arabidopsis, alfalfa, branchy grass, wheat, and *Nicotiana benthamiana*.

Some specific metabolites such as salicylic acid, ethylene, isoflavones, and γ-aminobutyric acid can prevent the growth and gene transfer efficiency of *Agrobacterium* (Nonaka et al. 2008; Hasan Nudin et al. 2015; Zhang et al. 2015; Park et al. 2010). Salicylic acid attenuates the kinase activity of the Vir A protein, which prevents the activation of vir genes (Yuan et al. 2007). On other hand, the addition of surfactants, polyamines, and amino acids to the culture medium has proven to improve TF positively. One of them, polyamines promote cell division, totipotency, and plant cell differentiation (Aydin et al. 2016; Jangra et al. 2022). The addition of polyamines to the transformation process leads to the induction of vir genes and T-DNA transfer, thereby increasing the efficiency of transformation (Xu et al. 2022). When 0.1 M spermidine was supplemented to the regeneration medium to help get transformants from pHK21/LBA4404-infected calli, the TF went up from 1.2% to 3.9% (Khanna and Daggard 2003).

Agrobacterium strain, *Agrobacterium* density, acetosyringone (AS) concentration, explant type, pre-culture process, co-cultivation conditions, antibiotics used in plant selection and against *Agrobacterium* growth, hormone types and concentrations used are all among the factors that affect gene transfer in wheat. For every transformation system, the optimum level of each of these components must be established. The most widely used *Agrobacterium* strains for wheat transformation include AGL0, AGL1, C58C1, EHA101, EHA105, GV3101, and LBA4404 (Hao et al. 2021; Upadhyaya et al. 2021; Ahansal et al. 2022; Sing and Kumar et al. 2022; Wang et al. 2022a, b, c). Khurana et al. (2002) used *Agrobacterium* LBA4404 to develop transgenic tetraploid wheat (*Triticum dicoccum Schuble*) plants from mature embryo-derived calli. Studies with diverse wheat explants revealed that a high *Agrobacterium* density boosted transitory GUS expression but was not related to a higher persistent transformation frequency (Cheng et al. 1997). Amoah et al. (2001) used *Agrobacterium* cells with a cell density of OD_{600} 1–1,5 and showed a significant rise in the number of explants showing high *β-Glucuronidase* (GUS) activity. According to Kumar et al. (2019), a bacterial density of $OD_{600 =}$ 1 resulted in the highest explant viability (83.6%).

Agrobacterium has a broad host range, infecting at least 41 plant groups, most of which are woody (De Saeger et al. 2021). Although cereals are not *Agrobacterium*'s native host, numerous investigations have been conducted to find host strains that are compatible with different wheat genotypes (Pérez-Piñeiro et al. 2012). Besides, AS has been used to induce *Agrobacterium* in wheat gene transfer studies. The phosphorylation of the VirG protein is triggered when the VirA protein is activated by phenolic chemicals such as AS. This in turn promotes the inducement of other *Vir* genes contained in the Ti plasmid (Chen et al. 2022a, b). Aadel et al. (2021) described that the addition of 0, 100, 200, and 400 µM AS to the *Agrobacterium* culture, especially of 200 µM AS increased the callus resistance frequency (44%) in the Achtar (from intact-immature cultured) wheat cultivar. Sing and Kumar (2021) successfully used 150 µM AS concentrations in several bread wheat genotypes such as HD 2967–3086 and Bobwhite for gene transfer studies. Kharb et al. (2022) supplemented the bacterial suspension with 200 µM AS during co-cultivation to facilitate the transformation of wheat and rice.

A dilemma in the planning of laboratory studies is the selection of mature or immature embryos. Successful studies of *Agrobacterium*-mediated transformation in wheat have been performed both using immature embryos and mature embryos to produce fertile transgenic plants (Kuluev et al. 2022; Liang et al. 2022; Sing and Kumar 2022). When we look at gene transfer studies with wheat tissue culture, it is understood that gene transfer to immature embryos is still the best approach. In contrast, gene transfer to tissues derived from mature embryos has a significantly low TF and can only be applied to a limited number of germplasms. Wang et al. (2009) transformed longitudinally cut mature embryos and found that the TF for the cultivars Bobwhite, Yumai 66, and Lunxuan 208 was, respectively, 0.06%, 0.67%, and 0.89%. Although immature embryos are frequently used for gene transfer studies in wheat, they are difficult to obtain throughout the year and their suitable stage for culture is severely limited (Yu et al. 2008; Ding et al. 2009). Therefore, correctly identifying the study material will increase the TF. However, endosperm-supported mature embryos had a larger percentage of embryogenic callus and a much higher rate of regeneration (Chauhan et al. 2017).

The next and another principal subject is the selection and concentration determination of surfactants. Surfactants such as Silwet, Pluronic F-68 -127, and Tween 20–80 have been reported to significantly affect T-DNA delivery during inoculation and co-cultivation of wheat transformation. Also, these surfactants are used for the hold of *Agrobacterium* to the surfaces of plant tissues in gene transfer. Pluronic F-68 (0.03% w/v) was added to the IM medium to increase transformation efficiency (Cheng et al. 1997). Researchers found that Pluronic F-68 worked well at 0.01% concentration (Ding et al. 2009). Parmar et al. (2015) evaluated the transformation efficiency of Pluronic F-68 at diverse doses (0, 0.015, 0.05, 0.1, 0.15, and 0.2%) in a gene transformation study in mature embryos. As a result, it was revealed that the maximum GUS expression was 0.15% of Pluronic F-68. Maximum Silwet L-77 concentrations of 0.05% were found to negatively impact the survival and callus formation of newly isolated immature embryos, leading to the determination that a concentration of 0.01% was optimal (Wu et al. 2003). In wheat gene transfer, 0.05% Silwet L-77 and 100 µM AS were prepared and added to the medium just before starting the study and successfully carrying out gene transfer (Hayta et al. 2021).

Drying can be vital to preventing some serious problems from occurring, but how?

After infection with *Agrobacterium*, it is essential to dry pre-cultured wheat embryos or embryogenic calluses before being placed in the co-cultivation medium. When using filter paper for co-cultivation, *Agrobacterium* overgrowth is limited, leading to an 82.3% explant survival rate. This compares to a survival rate of only 22.7% when using a medium-based method (Kumar et al. 2019). The effect of temperature on T-DNA delivery during co-cultivation is known. Bacterial conjugation studies determined that the most suitable temperature for T-DNA transfer was 19 °C (Salas et al. 2001). The ideal temperature for vir gene induction (25 °C) is typically lower than the ideal temperature for *Agrobacterium* vegetative development (28–30 °C) (Allahi et al. 2014). The co-cultivation temperature of monocot plants such as wheat is 24–25 °C, but the co-cultivation temperature of some crops reaches 28 °C (Opabode 2006). Following all transformations, the tissues are kept in the co-cultivation medium at 25 °C for 2–5 days (Wu et al. 2003; Zhao et al. 2006; Hayta et al. 2019). Transformed tissues frequently become sternly necrotic after co-cultivation during *Agrobacterium*-mediated transformation processes. It is known that this is because cultured wheat immature embryos rapidly produce H_2O_2 and that most tissues turn brown when these tissues come into contact with *Agrobacterium* (Tao et al. 2011). Browning and necrosis of infected tissues following *Agrobacterium* infection are significant barriers to gene delivery in wheat (Shrawat and Lörz 2006). Browning and necrosis tissues do not subsequently form somatic embryos and die quickly (Shrawat et al. 2007). Ascorbic acid, glutathione, lipoic acid, selenite, and cysteine are all antioxidants that have been shown to improve plant regeneration and TF while going through genetic transformation by reducing necrosis and darkening of the tissues (Jones et al. 2005; Dan 2008).

Using antibiotics does really help? If so, which one is the true one, and what is the effective dose of that?

For calli that are 4–20 days old, post-co-cultivation *Agrobacterium* removal or growth suppression using antibiotics is challenging (Kumar et al. 2017a, b). The dose of cefotaxime antibiotic used to kill *Agrobacterium* after gene transfer is very important. Cefotaxime is penicillin and contains the β-lactam group and lyses and kills the bacteria by inhibiting the biosynthesis of a component of the bacterial cell wall called peptidoglycan (Kumar et al. 2017a, b). It has the least adverse effect on most plant tissues. However, Ishida et al. (1996) found that high concentrations of antibiotics like cefotaxime were harmful to explants and reduced the transformation frequency by several folds. Li et al. (2002) stated that embryogenic callus could be encouraged on media with either 250 mg l^{-1} or 500 mg l^{-1} of cefotaxime, but that the frequency was much lower than on a medium without antibiotics. Before beginning the gene transfer, determine the lethal dose of cefotaxime on the plant based on the genotype to be used.

Herbicide resistance and antibiotic tolerance genes are often utilized as selectable markers in wheat transformation because they drastically decrease the escape rate. hpt II (hygromycin phosphotransferase) gene which confers hygromycin B antibiotic resistance (Sing and Kumar 2022), npt II (neomycin phosphotransferase II) gene which confers kanamycin antibiotic resistance (Mushke et al. 2019), bla (β-lactamase) gene which confers resistance to ampicillin antibiotic (Qin et al. 2022), EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene which confers glyphosate herbicide resistance (Arndell et al. 2019) and bar (bialaphos resistance) and pat (phosphinothricin acetyltransferase) genes which confer resistance to phosphinothricin herbicide (Thiyagarajan et al. 2022) were used commonly as selection marker genes.

Antibiotics in excessive amounts destroy non-transformed cells and slow the growth of transformed cells and plants, delaying the regeneration process. Interestingly, some of the wheat seeds in gene transfer studies initially germinate under antibiotic selection, but these plants do not survive any longer. The effectiveness of antibiotic selection is influenced by a variety of factors, including the species of plant, the types of tissue, the cultivar type, and organs present, and even the stages of development present within the same species (Tran and Sanan-Mishra 2015). Kanamycin and hygromycin have been effectively employed as selectable markers in the transformation of various legume crops. According

to Abdul et al. (2011) wheat cv. The GA-2002 experiments used the *Agrobacterium* strain (LBA4404) with the binary vector pBI121. Plant selection was done with 125 mg/l of kanamycin, and the resistant plants that were chosen were put through PCR testing and GUS histochemical staining. For this reason, before starting gene transfer studies in wheat, the doses of selective antibiotics to be used should be determined by preliminary trials.

Future perspective

Uncovering the mechanism of the immune system developed by bacteria against phages and plasmids has formed the basis of various genome editing tools (Mahler et al. 2022). Researchers are constantly working towards improving better technologies for making our lives easier. CRISPR/Cas is based on the natural protection mechanism found in bacteria and has been continuously developed since its emergence. Despite significant efforts by researchers, progress in the genetic engineering of wheat continues to lag other important agricultural crops such as maize and rice. This can be related to the genetic characteristics of wheat, such as its extremely large about 17,000 Mbp and complicated genome structure, and the significant dependence of most wheat species on growth and regeneration in vitro. It is thought that genetic engineering studies will mostly continue with CRISPR/Cas systems. Even though wheat's huge genome and complicated polyploid nature have previously slowed the progress of wheat genetic engineering, effective CRISPR/Cas9 systems are now accessible for the development of wheat biology (Li et al. 2020). NHEJ is plants' predominant repair pathway to create gene replacements and gene chains. However, the researchers believe that precision gene editing through HR will be used in wheat breeding soon by improving the efficiency of HR through some mechanisms. Strategies applied to increase genetic transformation efficiency in wheat go beyond simply optimizing culture medium composition and growth conditions. Understanding the problems of gene transfer to wheat is necessary for solving the related mechanisms with the developing genetic engineering tools. In addition, the researchers believe that precision gene editing through HR will be used in wheat breeding soon by improving the efficiency of HR through some mechanisms.

Recent advancements in CRISPR/Cas9 technology have led to the development of base editing and prime editing techniques that can more precisely modify the wheat genome. Although these techniques are relatively new, they show promise for improving wheat quality in the future. For instance, base editing could be used to introduce point mutations to specific genes that are associated with desirable traits, while prime editing could be used to make more complex edits to the genome without the need for double-strand breaks. Further research is needed to fully explore the potential of these techniques for improving wheat production.

Targeted genome editing has the potential to completely revolutionize plant breeding by allowing for the creation of wheat cultivars with improved resistance to environmental challenges generated by climate change. The use of the CRISPR/Cas9 system in drought stress tolerance or signaling engineering has not been enough studied in wheat but may demonstrate to be an important tool in the future to understand the functions of genes involved in signaling pathways. Additionally, when the studies on the nutritional quality of wheat are examined, it is understood that there are not enough studies on this subject, and in the future, researchers should accelerate their studies to raise the quality of the wheat grain to food the growing population and reduce hidden hunger.

Today, in many laboratories around the world, scientists are focused on understanding, developing CRISPR technology, and contributing knowledge to the "Science Pool", by this way, have begun to produce developments and patents to make it more effective and efficient every day.

All new developments have brought fear and criticism throughout human history. Does CRISPR technology also come with risks? What are the ethical issues? Can editing the gene pool lead to unpredictable results? Could CRISPR lead to off-target mutations in plants? What kind of damage can be caused at partially matched genomic loci through chromosomal rearrangements? If gene activity is lost, what physiological or signal abnormalities can occur? As plant scientists, while concentrating on these ideas, we also must listen to society, understand their concerns, and produce satisfactory answers. CRISPR is one of the most promising technologies for crop production, particularly given the widespread public concerns about GMO crops.

GMO and Genome Editing are fundamentally different methods. CRISPR technology is a good opportunity to tackle anti-GMO approaches. Small mutations such as deletions or insertions provide a great occasion for us to make changes to agronomic traits without leaving any foreign DNA behind. In the future, as society's needs change, plant breeders would increasingly use CRISPR resemble technologies instead of transgenic plants to produce high-quality and safe plants.

Global-scale evaluations should be made for the future of CRISPR technologies. As a part of the planned CRISPR goals, it is crucial that people living in low-income, low-R&D potential geographies be included so that they will have access to agricultural products (particularly essential nutrients like wheat) that are of sufficient quality and quantity in the future. There is also a need to establish a clear framework for all the legal regulations regarding intellectual property rights, R&D, production, and trade of CRISPR patents.

Considering agricultural purposes, CRISPR technology has the potential to lead to improvements in the extent of our obligations such as diversification of crops, improvement of their quality, herbicide tolerance, and resistance to diseases and pests through metabolic engineering and editing of host genes in plants. To achieve efficient genome editing, this technology must be improved, including the elimination of its current problems, such as low regulatory efficiency, loss of function genes, physiological or signal abnormalities. Insomuch, CRISPR technology is still considered to be in its infancy by some scientific assessments. More effective DNA-cutting tools and repairs removed from randomness are the goals for the renewed CRISPR technology of the future while eliminating random outputs for therapeutic effect is among the goals of developing the technique as well. As a result of these improvements, more complex targets would be achievable.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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