GENOME EDITING IN BREAD WHEAT USING CRISPR/CAS9

by

Alexander Dijkerman

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Supervisors:

Prof Johan Burger

Dr Manuela Campa

Mr Willem Botes

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Alexander Dijkerman

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Abstract

Bread wheat (*Triticum aestivum*) is one of the most important food crops consumed by humans, providing approximately 20% of the world's total caloric intake. However, wheat yields must be increased to supply the growing demand of an increasing global population. Traditional breeding techniques will not be sufficient to confront this challenge and improved genetic engineering and molecular-based techniques will be a necessity. The CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR associated) technology has emerged as a promising genetic engineering tool for the purposes of plant breeding. The CRISPR/Cas9 system has recently been developed as a DNA-free genome editing technique allowing for a precise and efficient method to genetically improve bread wheat while mitigating regulatory concerns. This study, therefore, aimed to establish CRISPR/Cas9 system in bread wheat and investigate the feasibility of this system in a DNA-free format. To achieve this, ribonucleoproteins (RNPs) were assembled by complexing single guide RNA (sgRNA) sequences targeting regions of a gene involved in the carotenoid pathway to the Cas9 protein. The RNPs were subsequently introduced to immature embryos through biolistic bombardment. Immature embryos were assessed to confirm successful editing of the target genes. No editing was detected in the experimental target. Evidence is provided for successful editing in embryos bombarded with RNPs mediated by a previously validated sgRNA. To establish an efficient method of achieving CRISPR/Cas9 edited wheat plants, a multiplex CRISPR/Cas9 DNA construct was assembled and introduced into wheat tissues through *Agrobacterium*-mediated transformation and particle bombardment. No edits were detected in plantlets that were regenerated on selective media from embryos transformed with *Agrobacterium* carrying the CRISPR/Cas9 construct. However, indels were detected in pre-initiated calli bombarded with the multiplex CRISPR/Cas9 DNA construct when analysed with the ICE v2 online software. Furthermore, various wheat transformation and regeneration protocols are assessed. Overall, the results provide insights into methods to deliver CRISPR/Cas9 components into bread wheat explant tissue for genome editing. A rapid and accessible method of screening for edits in pooled samples through PCR/RE assays followed by ICE v2 software analyses is demonstrated. Further, a vector delivery method that could circumvent challenging tissue culture procedures through the bombardment of mature embryos is explored. Possible optimisations of CRISPR/Cas delivery systems and experimental design are highlighted for future studies.

Opsomming

Broodkoring (*Triticum aestivum*) is een van die belangrikste voedselgewasse wat deur mense verbruik word, wat ongeveer 20% van die wêreld se totale kalorie-inname verskaf. Koringopbrengste moet egter verhoog word om in die groeiende aanvraag van 'n toenemende wêreldbevolking te voorsien. Tradisionele teeltegnieke sal nie voldoende wees om hierdie uitdaging die hoof te bied nie en verbeterde genetiese ingenieurswese en molekulêr-gebaseerde tegnieke sal 'n noodsaaklikheid wees. Die CRISPR/Cas-tegnologie het na vore gekom as 'n belowende genetiese ingenieurswerktuig vir die doeleindes van plantteling. Die CRISPR/Cas9-stelsel is onlangs ontwikkel as 'n DNS-vrye genoomredigeringstegniek wat voorsiening maak vir 'n presiese en doeltreffende metode om broodkoring geneties te verbeter terwyl regulatoriese bekommernisse versag word. Hierdie studie het dus ten doel gehad om CRISPR/Cas9-stelsel in broodkoring te vestig en die uitvoerbaarheid van hierdie stelsel in 'n DNS-vrye formaat te ondersoek. Om dit te bereik, is ribonukleoproteïene (RNP's) saamgestel deur enkelgids-RNA (sgRNA)-volgordes te ontwerp wat streke van 'n geen in die karotenoïedweg, teiken, aan die Cas9-proteïen te bind. Die RNP's is daarna in onvolwasse embrio's ingedra deur biolistiese bombardement. Onvolwasse embrios is geassesseer om suksesvolle redigering van die teikengene te bevestig. Geen redigering is in die eksperimentele teiken bespeur nie. Bewyse word egter verskaf vir suksesvolle redigering in embrios wat gebombardeer is met RNP's wat vooraf-bevestigde sgRNA bevat het. Om 'n doeltreffende metode te vestig om CRISPR/Cas9 geredigeerde koringplante te genereer, is 'n multipleks CRISPR/Cas9 DNA-vektor saamgestel en in koringweefsels ingevoeg deur *Agrobacterium*-gemedieerde transformasie en partikelbombardement. Geen redigering is opgemerk in plantjies wat op selektiewe media geregenereer is vanaf embrios wat getransformeer is met *Agrobacterium* met die CRISPR/Cas9-konstruk nie. Indels is egter opgespoor in vooraf-geïnisieerde kallusse wat gebombardeer is met die multipleks CRISPR/Cas9 DNA-konstruksie wanneer dit met die ICE v2 aanlyn sagteware ontleed is. Verder word verskeie koringtransformasie- en regenerasieprotokolle geassesseer. Oor die algemeen verskaf die resultate insig in metodes om CRISPR/Cas9-komponente in broodkoring ex-plantweefsel vir genoomredigering te lewer. 'n Vinnige en toeganklike metode van sifting vir redigering in saamgevoegde monsters deur PCR/RE-toetse, gevolg deur ICE v2-sagteware-ontledings word gedemonstreer. Verder word 'n vektor-afleweringsmetode ondersoek wat uitdagende weefselkultuurprosedures kan omseil deur die bombardement van volwasse embrios. Moontlike optimalisering van CRISPR/Cas-afleweringstelsels en eksperimentele ontwerp word uitgelig vir toekomstige studies.

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List of abbreviations

bp	basepair
CAF	Central analytics facility, Stellenbosch University
Cas	CRISPR-associated protein
CRISPR	Clustered regularly interspaced short palindromic repeat
crRNA	CRISPR RNA
DAP	days after pollination
DNA	Deoxyribonucleic acid
DSB	double-strand break
dsDNA	double-stranded DNA
EMS	ethylmethanesulfonate
GE	Genome engineering
gRNA	guide RNA
GUS	β-glucuronidase
HDR	homology-directed repair
InDels	insertions or deletions
IVT	*in vitro* transcript
LB	Luria-Bertani
LCYB	Lycopene β-cyclase
MAS	Marker-assisted-selection
ME	mutagenesis efficiency
MES	2-(N-morpholino) ethanesulfonic acid
MGE	multiplex genome editing
mRNA	messenger RNA
MS	Murashige and Skoog
NHEJ	non-homologous end-joining
nt	nucleotide
PAM	protospacer-adjacent-motif
PCR	Polymerase chain reaction
PCR/RE	PCR/Restriction enzyme assay
PEG	Polyethylene glycol
RE	Restriction enzyme
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SAM	shoot apical meristem
SDN	site-directed nuclease
sgRNA	single guide RNA
SNP	single nucleotide polymorphism

TALEN	transcription activator-like effector nuclease
TECCDNA	Transient Expression of CRISPR/Cas9 DNA
TECCRNA	Transient Expression of CRISPR/Cas9 RNA
TF	transformation frequency
TILLING	Targeting Induced Local Lesions in Genomes
tracrRNA	trans-activating CRISPR RNA
ZFN	zinc-finger nuclease

Chapter 1: Introduction

1.1 Background

Bread wheat (*Triticum aestivum L*.) is one of the most important crops in the world, providing more than 30% of the total calories consumed worldwide [1]. Due to its adaptability to various climates, wheat is cultivated almost globally. Despite significant increases in wheat production in the past decades following the "Green revolution" in the 1960s [2], it will need to address the upcoming challenges of an increasing global population, climate change, and water shortages in arid and semi-arid lands [3]. Moreover, these challenges must be met through substantial yield gains since available land for agricultural expansion is ever limited [4]. The current rates of yield increases are predicted to be insufficient to reach the goal of a 50% increase in crop production by 2050 [5,6]. Furthermore, genetic approaches to select for desirable phenotypes in wheat is time-consuming due to wheat's hexaploid genome and gene function redundancy [7].

Genome editing technologies have recently emerged as revolutionary tools for crop improvement. The clustered regularly-interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has been used to elicit targeted mutations in a variety of organisms [8,9]. Its efficiency, robustness, simplicity, and its adaptability to target multiple genes has seen it become the most popular genome editing tool in use [10]. The application of CRSIPR/Cas9 strategies requires the DNA sequences of target sites. Given the recent availability of the wheat reference genome RefSeq v.1.0 [11], the CRISPR/Cas9 system could become increasingly useful in genomic studies of wheat. Additionally, CRISPR/Cas9 permits researchers to target all homoalleles of a gene simultaneously and further supports the targeting of multiple sites. CRISPR/Cas9 therefore holds great potential in the functional characterisation of genes affecting agronomic traits in polyploid wheat. Since its first reported use in wheat, CRISPR/Cas9 has been used to target numerous genes of agronomic and scientific interest, such as *TaGW2* to increase grain weight [12], α-gliadin genes for lowered gluten content [13], and *TaCENH3* for haploid induction [14].

The CRISPR/Cas9 system consists of two components: a Cas9 endonuclease and a single guide RNA (sgRNA). For the purpose of genome editing, these components are delivered into host cells through either expression plasmids, *in vitro* transcripts (IVTs), or preassembled ribonucleoproteins (RNPs) [15]. Typically, CRISPR/Cas9 DNA is delivered through a vector carrying the CRISPR/Cas9 cassette through *Agrobacterium*-mediated transformation or particle bombardment [16]. This is commonly done to achieve stably transformed plants. However, CRISPR/Cas9 DNA constructs can be delivered to wheat cells to act transiently as has been shown in a recent study [17]. While in 2017, researchers demonstrated a novel strategy of introducing CRISPR/Cas9 components into wheat cells in the form of RNPs [18]. Since this approach uses no DNA, the researchers were able to regenerate genome-edited wheat plants containing no foreign DNA sequences.

1.2 Aims and objectives

The current study comprised of two aims; 1) To establish a DNA-free genome editing platform in bread wheat using CRISPR/Cas9 ribonucleoproteins, and 2) To establish an efficient genome editing method in bread wheat via CRISPR/Cas9.

To achieve these aims, the following objectives were formulated. For the first aim:

i. To design and generate sgRNAs targeting conserved regions across all three sub-genomes of bread wheat.
ii. To assemble a functioning CRISPR/Cas9 ribonucleoprotein *in vitro*.
iii. To introduce the CRISPR/Cas9 ribonucleoprotein into wheat tissue.
iv. To determine if successful editing has occurred in target tissue.

For the second aim:

i. To assemble a CRISPR/Cas9 expression construct containing multiple sgRNA target sequences.
ii. To introduce the multiplex CRISPR/Cas9 construct into wheat tissue.
iii. To determine the efficacy of the multiplex CRISPR/Cas9 construct in eliciting targeted edits in wheat.

1.3 Overview of chapters

Chapter 2 provides a review of the existing literature covering earlier and recent methods to genome engineer bread wheat. Different strategies on crop improvement, and mechanisms for CRISPR/Cas9 component delivery are evaluated. Further an overview of CRISPR/Cas9 applications in bread wheat is provided.

In **Chapter 3**, CRISPR/Cas9 ribonucleoproteins are assembled and delivered to immature wheat embryos through particle bombardment. Bombarded embryos are subjected to early assessment to determine if successful DNA-free editing occurred.

Chapter 4 describes the introduction of a CRISPR/Cas9 DNA construct into explant wheat tissue using different approaches. Different explants are assessed and regeneration media protocols and delivery strategies. Furthermore, explants are assessed for successful editing.

Chapter 5 summarises the findings of the research project and provides considerations for future practices.

Chapter 2: Literature review

The improvements of crops has been practiced by farmers through phenotypic selection for thousands of years, while more specific improvements to crops through the selection of specific traits has been practiced since the beginning of the 20th century [19]. Over the past five decades, the increasing availability of novel molecular techniques has allowed breeders to select for specific beneficial genes which has resulted in huge advancements in crop breeding.

The technologies behind these advancements are constantly changing and improving. To utilise these techniques, however, it is important to understand their advantages and limitations. The purpose of this literature review is therefore to provide an overview of modern strategies in crop improvements with special emphasis on the most popular current strategy for targeted mutagenesis, the CRISPR/Cas9 system. Various applications of the CRISPR/Cas9 system in bread wheat is reviewed and the methods used to elicit CRISPR/Cas9-mediated editing is described.

2.1 Wheat Genome

Bread wheat (*Triticum aestivum L.*) was domesticated 8000 years ago and has subsequently undergone hybridisation and genome duplication events, resulting in its current allohexaploid genome (2n = 6x =42, AABBDD) [20]. The three separate sub-genomes, A,B and D, are derived from three ancestral diploid species that diverged between 2.5 and 6 million years ago [21]. This accounts for bread wheat's mammoth genome of 16 Gb, five times the size of the human genome and 40-fold larger than that of rice [22]. Furthermore, the genome consists mainly of repetitive DNA elements (85%) [23], while the most recent estimates suggest the presence of over 107 000 genes [11]. Due to its ploidy level, the wheat genome presents a high degree of functional redundancy since each gene typically exists as three copies that, on average show over 95% similarity across their coding regions [24]. These complex characteristics of the wheat genome are in part responsible for the relatively slow development of genetic applications in wheat [25]. The rate of research in the crop is further limited by its relative recalcitrance to genetic transformation and *in vitro* culture [26] as well as its long regeneration time and poor regeneration ability [27]. Ongoing efforts to sequence the bread wheat genome first resulted in a draft genome sequence in 2014, until the recent publicly available chromosome-scale assembly IWGSC RefSeq v1.0 genome sequence [28,29]. These advancements have allowed researchers to further reveal the genomic structure of bread wheat with the recent genome assemblies of 10 bread wheat cultivars [30], while more chromosome-scale assemblies of different cultivars continue to be released [31]. Further refinements have recently been made to the v1.0 assembly with inconsistencies being resolved and sequence gaps filled for 10% of the assembly, while the accuracy of the remaining 90% has been validated [32]. The IWGSC Annotation v2.1 is now available at https://wheat-urgi.versailles.inrae.fr/Seq-Repository/Annotations. These resources should greatly facilitate research henceforth.

2.2 Approaches to crop improvement

2.2.1 Conventional breeding and Marker-assisted-selection

Conventional crop breeding is based on hybridisation and phenotypic selection, relying on the development of new desirable traits through recombination [33]. For centuries, phenotypic traits were selected by farmers without knowledge of the underlying genetic mechanisms affecting the traits. Commercial plant breeding involving artificial and deliberate cross-pollination became common in the early 20th century [34]. Despite the trait improvements gained through breeding, the complexities of allelic effect on the traits being selected for were not deeply understood until the advent of DNA sequencing [35,36]. The ability to sequence specific regions of DNA permitted the utility of molecular markers to swiftly improve varieties [37,38]. Marker-assisted-selection (MAS) [19] has since significantly accelerated the crop breeding process. By using molecular markers that are linked with beneficial traits, the necessity to collect phenotypic data for quantitative or challenging traits can be mitigated. MAS has been applied successfully to wheat, for example, to select for quality traits more efficiently [39]. However, successful breeding programs depend on the introduction of allelic diversity which is influenced by the frequency of recombination [40]. Recombination allows breeders to remove a beneficial allele from its genetic background, which may possess other alleles with opposing effects. The recombination rates in certain parts of the genome are often suppressed, rendering beneficial alleles inaccessible to selection [41,42]. Furthermore, utilising wild ancestors of wheat as a source of genetic variation is limited by a reduction in recombination resulting from genetic divergence [43]. Therefore, despite the effectiveness of MAS at accelerating the crop breeding process, it is often limited to selecting for variation present in the germplasm of wheat.

2.2.2 Random mutagenesis

The process of selection, in developing commercial wheat cultivars, has resulted in a loss of genetic diversity [44]. This paucity of genetic variation represents a bottleneck in crop improvement [45]. In response, several techniques, including mutation breeding, emerged in the 20th century [46]. Using chemical and physical mutagens such as ethylmethanesulfonate (EMS) or radiation, genetic variability can be rapidly generated to develop new plant varieties. Induced mutagenesis greatly expanded and accelerated crop breeding efforts with numerous crop varieties having been developed to date; the FAO/IAEA Mutant Variety Database currently contains over 3300 officially released mutant varieties from nearly 230 plant species (http:// mvd.iaea.org/). Radiation-based mutagenesis typically elicits large deletions and translocations in the target crop, while EMS causes G/C to A/T conversions in the genome. The point mutations induced through EMS treatment are more desirable in breeding programs as they are less likely to reduce plant viability than large deletions. Thus, EMS treatment is the most widely used mutagenic method [47]. The development of Targeted Induced Local Lesions in Genomes (TILLING) provides a simple molecular platform to identify induced mutations in a target gene, compared to observing the phenotypic effects of a mutation [48]. Typically, a large number of EMS-treated wheat plants will be screened for premature stop codons present in a gene of interest [49]. However, since bread wheat contains a hexaploid

genome, phenotypic effects caused by mutations on one genome can be concealed [50]. To obtain double or triple knockout-mutants therefore requires a series of crosses, which is a time-consuming process [47]. Furthermore, despite continued popularity, induced mutagenesis suffers a major drawback in that it engenders random mutations simultaneously across the entire genome which, if detrimental, subsequently requires extensive back-crossing procedures to eliminate [51].

2.2.3 Genetic transformation

In the 1980s, recombinant DNA methods employing *Agrobacterium tumefaciens* [52] and particle bombardment [53] were developed that allowed for the introduction of extrinsic genes of interest into target organisms. Since its emergence, numerous crops have been modified with performance-enhancing traits through transgene introgression. Although the genetic transformation of tobacco plants were reported by the mid-80's [54], the first transgenic wheat plants were obtained only in 1992. Vasil *et al.* [55], successfully produced fertile herbicide-resistant wheat lines using particle bombardment at a transformation frequency (TF) of about 0.2%. Following this landmark report, numerous efforts were attempted to improve the bombardment method and efficiency [56–58]. While one study reported a TF of over 60% in the wheat line Bobwhite [59], typical TFs achieved through particle bombardment-mediated transformation of wheat tissues remain low (see [60] for a detailed review). Cheng *et al*., [61] were the first to show that wheat can also be successfully transformed using *Agrobacterium*, obtaining TFs of up to 4.3% depending on explant tissue used. Until recently, gene transfer to wheat has been accomplished by numerous labs using particle bombardment- or *Agrobacterium*-mediated methods (with slight modifications) with TFs rarely exceeding 5% [62]. In 2014, the PureWheat protocol was developed by the Japan Tobacco Company with the authors claiming that a TF of up to 90% is achievable in certain cultivars [63]. In the years following, many studies employing the *Agrobacterium*-mediated PureWheat technique have reported TFs ranging from 37-45% [64,65].

Although traditional gene transfer allowed for more specific control over mutations induced and characteristics inferred, there are limitations to its application. Since the introduced gene is integrated at a random location in the genome, it could influence the expression of the host's native genes. Furthermore, the location where the gene is integrated in the genome also determines how effectively the transgene is expressed. Additionally, extensive assimilation of transgenic crops has been largely obstructed due to public perceptions regarding the safety of crops carrying foreign genes to human beings and concerns over unintended environmental effects. Consequently, technologies that can avoid the integration of foreign genes into host crops and are free of any foreign DNA are expected to be more publicly palatable and gain regulatory acceptance more rapidly. In a South African context, it should be noted that the South African authorities have, as of October 2021, decided that New Breeding techniques (NBTs) be classified under the amended GMO act of 2006 (Act No. 23 of 2006). These NBTs include the use of genome editing technologies, and derived

products will therefore fall under the regulatory framework of the GMO act (The full regulatory document can be found at https://www.dalrrd.gov.za/).

2.2.4 Site-Directed Nucleases

The recent emergence of engineered site-directed nucleases (SDNs) allows for the introduction of mutations at precise locations in the genome. To date, four iterations of site-directed nucleases have been used in plant research: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly-interspaced short palindromic repeats (CRISPR)-associated (Cas) endonucleases.

Mutagenesis by SDNs is based on the process of introducing double-strand breaks (DSBs) at a specific genomic site determined by the DNA-binding domain. These targeted DSBs are subsequently repaired by one of two primary endogenous repair mechanisms of the cell [66,67]. The cell's predominant repair mechanism is the error-prone non-homologous end-joining (NHEJ) pathway. Repair by NHEJ frequently involves small insertions or deletions (InDels) at the cleavage site that may generate a knockout mutation if the InDel results in a frameshift mutation or introduces a premature stop codon in the target gene [67,68]. Alternatively, DSBs can be repaired by the precise, but much less frequent, homology-directed repair (HDR) pathway. The HDR pathway uses homologous DNA sequences as templates for repair, and, by supplying an exogenous repair template, HDR can be exploited to precisely edit a genomic sequence or insert exogenous DNA [69,70].

Meganucleases are naturally occurring endonucleases that recognise specific DNA segments ranging from 12-40 bp in length, upon which they induce DSBs [71] (Figure 2.1). Hundreds of different types of meganucleases, containing unique recognition domains, have been identified [72]. Given the relatively large target site, meganucleases are highly specific. However, they are limited by their predetermined target sites and are further cumbersome to redesign for different target sequences [73]. As a result, they have seen limited use in plant research [74].

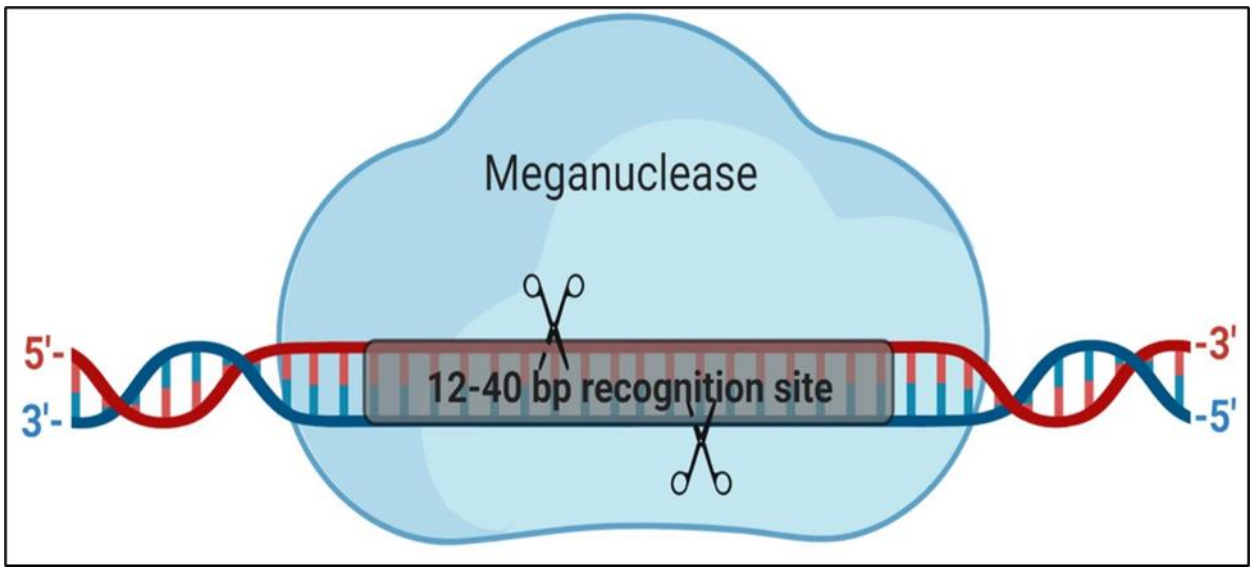


Figure 2.1. Site-specific meganuclease with innate DNA-binding domain. This figure was created using Biorender (https://biorender.com/).

ZFNs and TALENs are chimeric proteins comprised of the *Fok*I endonuclease fused to the DNA-binding domain of a Zinc finger or Transcription Activator-like Effector, respectively [75,76] (Figure 2.2a and b). The target sequences of ZFNs and TALENs are determined by protein-DNA interactions while the *Fok*I endonuclease, present as a dimer, generates DSBs [76]. ZFNs are limited by their relative inflexibility in possible target sites [74]. TALENs, on the other hand, are more versatile [77], and have found extensive use in crop plants including soybean [78] and barley [79]. TALENs have further been successfully utilised to knockout the TaMLO gene in hexaploid wheat conferring broad-spectrum resistance to powdery mildew [80]. Despite the potential promised by these initial genome editing tools, they have found limited use as they are based on complex protein-DNA interactions [81].

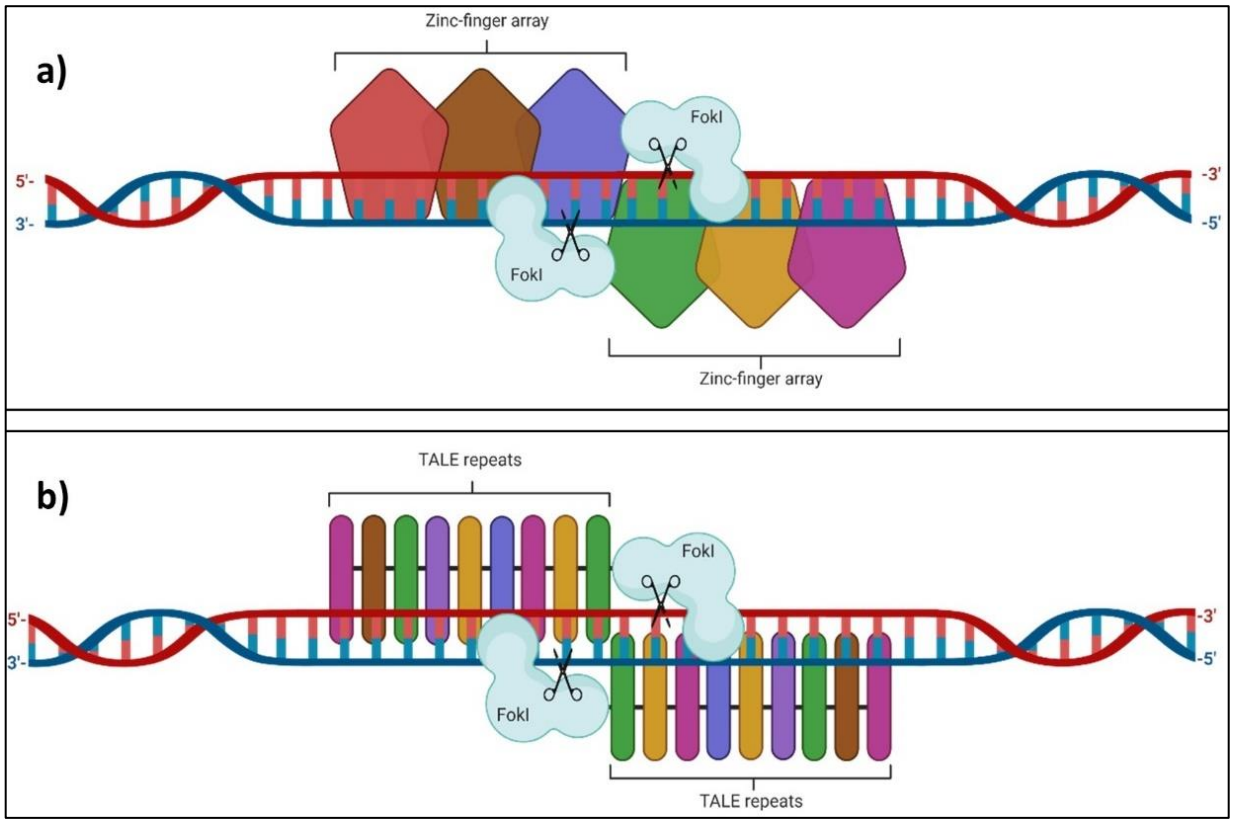


Figure 2.2. Site-specific endonucleases with programmable DNA-binding domains: a) Zinc finger nucleases (ZFNs) and b) Transcription activator-like effector nucleases (TALENs). This figure was created using Biorender (https://biorender.com/).

2.2.5 CRISPR/Cas9

In 2012, the emergence of the latest iteration of SDNs, the CRISPR/Cas system, rapidly transformed crop biotechnology**.** The source of the RNA-guided endonucleases is derived from the CRISPR/Cas adaptive immune system of prokaryotes. The CRISPR/Cas system in bacteria and archaea provides resistance against invading foreign genetic elements such as viruses and plasmids (Figure 2.3). Upon entry of the foreign elements, the adaptive immune system incorporates fragments of the invading nucleic material (known as spacers) into a CRISPR array located on the host genome. Upon subsequent infections by the same, or similar, foreign elements, CRISPR RNAs (crRNAs) complementary to the invading nucleic material are transcribed from the CRISPR array. The crRNA hybridises to a second RNA, the trans-activating CRISPR RNA (tracrRNA), consisting of base repeats that allows the crRNA-tracrRNA hybrid to complex to the Cas endonuclease and direct it to a specific target site to cleave the foreign nucleic acid, thus silencing the invading threat [82].

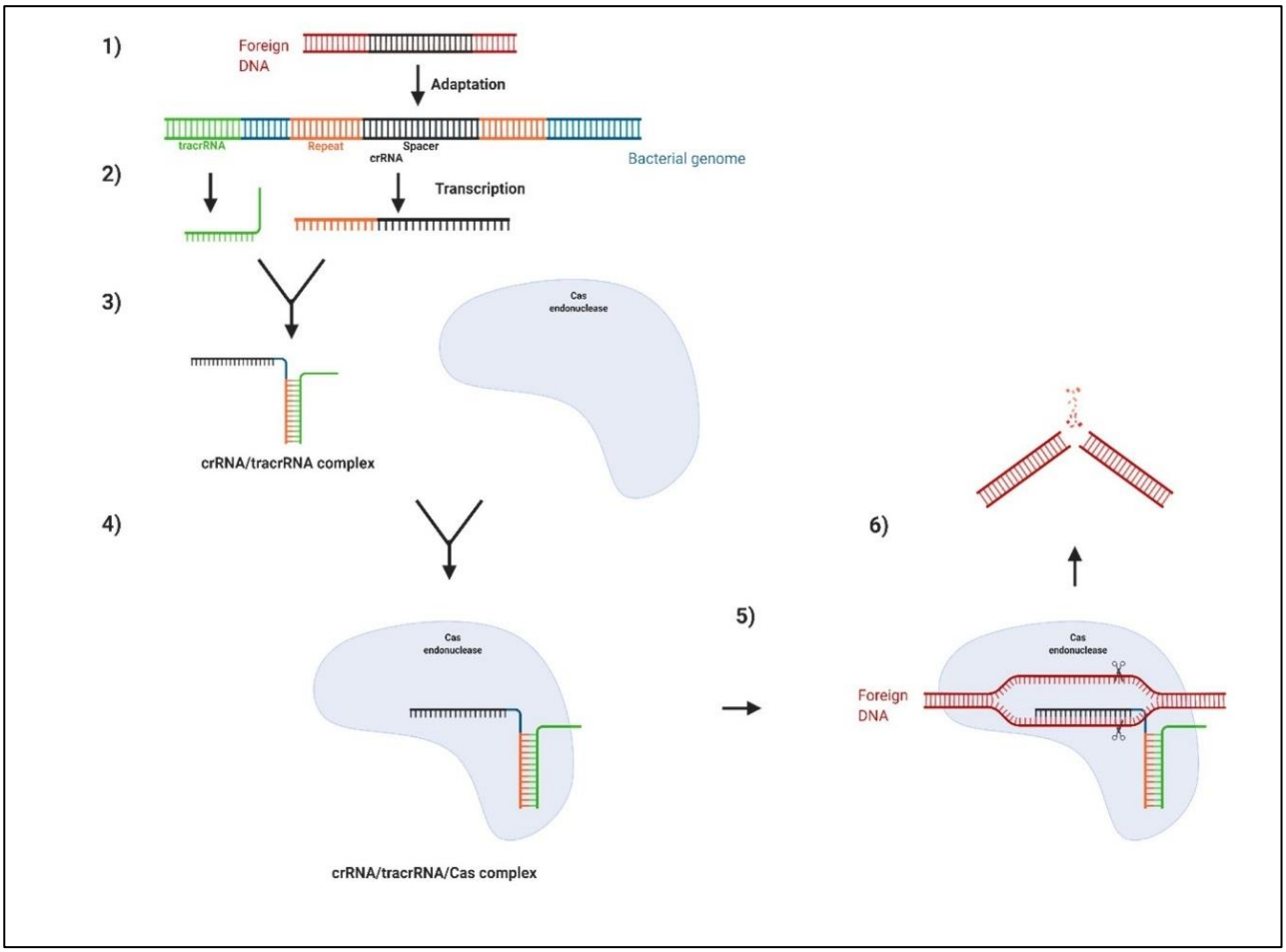


Figure 2.3. The CRISPR/Cas adaptive immunity response present in bacteria and archaea. 1) Upon the initial infection from foreign DNA, adaptation is initiated, and small fragments of the foreign DNA are integrated as spacers into CRISPR arrays in the host genome. 2) CRISPR array RNA (crRNA and tracrRNA) are transcribed and 3) are complexed. 4) The hybridised crRNA/tracrRNA complex subsequently binds to the Cas endonuclease. 5) Upon infection from similar foreign genetic sequences, the crRNA/tracrRNA/Cas endonuclease complex is directed to a specific target site on the foreign genetic sequence and cleaves it. 6) Cleavage results in the degradation of the foreign DNA and silencing of the intruding threat. This figure was created using Biorender (https://biorender.com/).

CRISPR/Cas systems are categorised into six (I-VI) types [83,84]. The type II CRISPR/Cas9 system, which uses the Cas9 endonuclease from *Streptococcus pyogenes* (called SpCas9), is currently the most widely employed in research applications. To wield the CRISPR/Cas9 system for genome editing, the crRNA and tracrRNA can be artificially fused together to generate a single guide RNA (sgRNA). The sgRNA complexes to the Cas9 endonuclease and directs it to a target sequence for cleavage. For cleavage to occur, the target sequence must lie directly 5' to a protospacer-adjacent-motif (PAM), which is recognised by the Cas protein. For SpCas9, the PAM sequence is represented by NGG [85] (

Figure 2.4).

The relative simplicity of the CRISPR/Cas9 system lies in the principle that the 20-nt target sequence can be changed to target any desired sequence given that the sequence is adjacent to a PAM site. Following the CRISPR/Cas9 system's validation in *Arabidopsis,* it has been successfully used for gene editing in numerous crop plants including maize [86,87], barley [88], rice [16,89], soybean [90], and wheat [16,18,80,91]. Owing to its relative simplicity and robustness, the CRISPR/Cas9 system

has rapidly usurped other SDNs to become the most popular genome editing technology currently in use [92].

2.3 CRISPR/Cas9 applications in wheat

In 2012, shortly after the original CRISPR/Cas9 principle was published, two independent research groups demonstrated the CRISPR/Cas9 system's applicability in the complex genome of wheat. Upadhyay *et al*. [91] directed Cas endonucleases to target two separate genes, *TaPDS* and *TaINOX*, simultaneously in wheat suspension cultures. Mutations were confirmed in up to 22% of sequenced amplicons. Meanwhile, Shan *et al*. [93] introduced CRISPR/Cas9 constructs into wheat protoplasts that were designed to target the *TaMLO* gene and achieved a mutagenesis efficiency (ME) of 28.5%. The group also showed that TALEN-induced *TaMLO* knockout mutant plants displayed partial resistance to powdery mildew, a disease that results in substantial yield loss. These studies were, however, restricted to only providing proof that the CRISPR/Cas9 system could be utilised to edit the polyploid genome of wheat since no plants could be regenerated from the explant tissues. Wang *et al*. [80] extended on these early reports, using immature embryos as explant material, to present the first mutant wheat plants developed by CRISPR/Cas9 technology. By simultaneously knocking out all the *TaMLO* homoalleles using target regions that are conserved on all three homeologs, they were able to regenerate mutant wheat plants that exhibited partial resistance to powdery mildew with a reported ME of 5.4%.

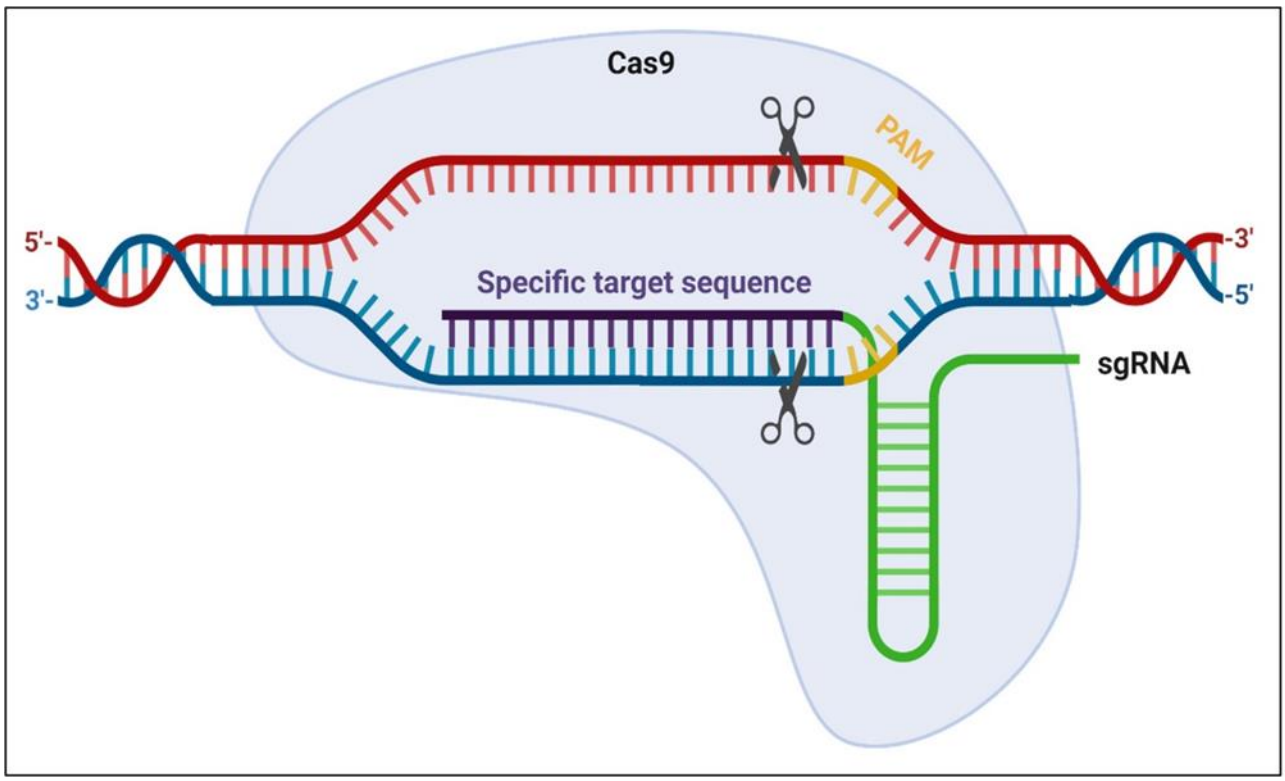


Figure 2.4. Site-specific DNA cleavage by the Cas9 nuclease mediated by complementarity between a single guide RNA (sgRNA) and the specific target sequence upon the presence of the protospacer-adjacent motif (PAM) (in yellow). This figure was created using Biorender (https://biorender.com/).

Since being validated in wheat tissues, CRISPR/Cas9 has predominantly been used to investigate the function of genes that influence commercial and agronomic traits in wheat. While some notable examples of the CRISPR/Cas9 applications are presented in detail in this section, an extensive listing of thus far published studies is provided in Table 2.1.

Following the previously published report that the *TaGW2* orthologue in rice acts as a negative regulator of grain size and thousand-grain weight, Wang *et al*. [94] were able to confirm the corresponding function of the *GW2* gene of wheat using Cas9 to produce knockout mutants. By generating all possible mutant combinations, the authors found that mutations in individual *TaGW2* homeologues significantly increased the size of the wheat grain. They further concluded that all three homeologues have an additive effect on grain size.

Wheat grains contain gluten proteins, which trigger certain pathologies in approximately 2% of the global population [95]. Gluten sensitivity is primarily associated with an immune response to the α-gliadin protein family, which are encoded by 45 genes in the wheat genome. Researchers recently designed two sgRNAs targeting sequences coding for α-gliadin proteins and were able to successfully produce low-gluten wheat lines that displayed an 85% decrease in immunoreactivity [13].

Hybrid crop varieties are often present with enhanced traits compared to homozygous parents due to heterosis [96]. Hybrid breeding has been widely used to produce major cereal crops such as maize [97] and more recently, rice [98]. Experimental wheat hybrids have exhibited yield advantages of up to 20% above elite wheat cultivars, as well as better responses to abiotic and biotic stresses [99,100]. Commercial-scale production of wheat hybrids, however, has been challenging, largely because of wheat's strong inbreeding nature. Consequently, only about 1% of the current world wheat area is used to cultivate hybrids [101]. Hybrid seed production in wheat can be dramatically facilitated by the development of male-sterile and double haploid plants. Employing a Cas9-mediated knockout approach, Singh *et al*. [102] generated triple homozygous wheat mutants in the *TaMS45* gene. Mutants aborted pollen development resulting in male sterility. More recently, frameshift mutations were introduced into the *TaMS1* gene using CRISPR/Cas9. The knockout mutants exhibited complete male sterility [103]. The haploid induction rate has been shown to increase by up to 6-fold by knocking out *ZmDMP* in maize [104], while in rice *OsMATL* knockouts led to a 2-6% haploid induction rate [105]. Researchers recently designed a sgRNA to knock out *TaPLA* homeologs on the A and D sub-genomes of wheat, reportedly achieving a haploid induction rate of 2-3% [106]. The *MTL* gene encodes a pollen-specific phospholipase. Editing of the *MTL* orthologs was shown to trigger haploid induction in maize [107] and rice [108]. Liu *et al.* [109] targeted all three *TaMTL* homeologues simultaneously using two sgRNAs and were able to achieve a haploid induction rate of up to 31.6%. Heterosis is often lost in subsequent generations due to genetic segregation. The CRISPR/Cas9 system was recently used to preserve heterosis in rice [110]. Researchers manufactured a genotype termed *MiMe* (Mitosis instead of Meiosis) by knocking out genes involved

in meiosis. The group then combined the *MiMe* genotype with haploidy by targeting the *OsMATL* gene and were able to produce clonal progeny that displayed the parental heterozygosity. The study demonstrates the applicability of using CRISPR/Cas9 technology to develop asexually reproducing crops.

Table 2.1 Survey of published studies utilising Cas9-mediated genome editing.

Target gene	Gene function	Phenotype	Explant tissue	ME (%)	Method	Purpose of study	Date	Reference
TaMLO	encode proteins that repress defenses against powdery mildew diseases	Powdery mildew resistance	Protoplast	28.5	PEG-mediated	Proof of concept	2013	[93]
TaINOX	Encodes for inositol oxygenase	Not measured	Suspension cells	22	*Agrobacterium*	Multiplex gene targeting method establishment	2013	[91]
TaPDS	Encodes PDS, a carotenoid pathway enzyme							
TaMLO	encode proteins that repress defenses against powdery mildew diseases	Powdery mildew resistance	Immature embryo	5.4	Biolistics	Powdery mildew resistant wheat plants	2014	[80]
TaGASR7	Involved in the control of grain weight and length	Increased thousand-grain weight	Immature embryo	9.5	Biolistics	Transgene-free wheat mutants	2016	[17]
TaDEP1	regulator of wheat growth and development	Dwarf wheat plants		Not reported				

TaLOX2	Associated to storability of wheat grain	Not measured						
TaNAC2	Regulates shoot branching							
TaPIN	Auxin-dependent adventitious root emergence							
TaGW2	Negative regulator of kernel width and weight in bread wheat							
TaGW2	Negative regulator of kernel width and weight in bread wheat	Not measured	Immature embryo	4.4	Biolistic with RNP	RNP bombardment method establishment	2017	[18]
TaPDS	Encodes PDS, a carotenoid pathway enzyme	No phenotype observed	Immature embryo	16.7	*Agrobacterium*	method establishment	2018	[111]
Alpha-gliadin family	associated with gluten sensitivity	decreased alpha-gliadin levels	Immature embryo	Not reported	Biolistics	decreased alpha-gliadin wheat	2018	[13]
TaMs45	encodes a strictosidine synthase-like enzyme required for male fertility	Triple KO male sterile	Immature embryo	Not reported	*Agrobacterium*	male sterility	2018	[102]

TaGW2	Negative regulator of kernel width and weight in bread wheat	Additive effect on grain size	Immature embryo	Not reported	Biolistics	increased grain size	2018	[94]
TaGW2	Negative regulator of kernel width and weight in bread wheat	Increased thousand-grain weight	Immature embryo	19.6	Biolistics	Increased grain size	2018	[112]
TaLpx1	facilitates infection by fusarium graminearum	Powdery mildew resistance						
TaMLO	encode proteins that repress defenses against powdery mildew diseases	Not measured		Not reported		fungi resistance		
TaPinb	major gene controlling grain hardness	Not measured	Protoplast	3.86-4.57	PEG-mediated	grain hardness	2018	[113]
TaWAXY	involved in synthesis of amylose			1.74-2.56		starch composition		
TaDA1	negative regulator of seed and organ size		Immature embryo	54.17	*Agrobacterium*	grain size		

TaPLA	Associated with sexual reproduction	Haploid wheat plants	Immature embryo	Not reported	*Agrobacterium*	Establishing haploid induction in wheat	2019	[106]
TaMs1	Male fertility	male sterility	Immature embryo	4	*Agrobacterium*	male sterility	2019	[103]
TaGW7	Encodes TRM, involved in cell division during tissue formation	increased width and weight and decreased length	Immature embryo	Not reported	Biolistics	Gene function in grain morphology	2019	[114]
TaCKX2-1	Associated with cytokinin metabolism	Increased grain number per spikelet	Immature embryo	5.2	*Agrobacterium*	method establishment	2019	[115]
TaGLW7	Positive regulator of grain weight	Not measured						
TaGW2	Negative regulator of kernel width and weight in bread wheat			Not reported				
TaGW8	Positive regulator of grain weight							
TaQsd1	Controls seed dormancy	extended seed dormancy	Immature embryo	Not reported	*Agrobacterium*	Reduce pre-harvest sprouting of wheat grains	2019	[116]
TaMTL	Pollen-specific phospholipase	Haploid induced wheat plants	Immature embryo	57.5	*Agrobacterium*	Establish efficient double haploid technology in wheat	2019	[109]

TaWAXY	involved in synthesis of amylose	Not measured		26.4-54		Improve starch quality of wheat		
TaNFXL1	Ortholog represses bacteria resistance in *Arabidopsis*	Increased resistance to *Fusarium graminearum*	Callus tissue	Not reported	Biolistics	Gene function in bacterial resistance	2019	[117]
TaNP1	encodes a putative glucose-methanol-choline oxidoreductase	No pollen produced	Immature embryo	Not reported	Biolistics	Male sterility	2020	[118]
TaMs2	Controls complete male sterility	Restored male sterility	Immature embryo	2.3-8.27	*Agrobacterium*	Restoring male sterility in wheat lines present with agronomic traits	2020	[119]
TaQ	Involved in spike development	Changed spike phenotype, plant height and flowering time	Immature embryo	8.9-33-3	*Agrobacterium*	Gene function in spike development	2020	[120]
TaCENH3	Involved in zygotic centromere formation	Haploid induced wheat plants	Immature embryo	Not reported	Biolistics	Establish efficient haploid technology in wheat	20202	[14]
TaSBEIIa	Implicated role in starch property	Modified starch composition	Immature embryo	Not reported	Biolistics	Increased resistant starch and amylose content	2020	[121]

TaARE1	Involved in nitrogen assimilation	Increased nitrogen starvation tolerance	Immature embryo	Not reported	Biolistics	Development of nitrogen efficient wheat	2021	[122]
TaIPK1	Involved in the phytic acid biosynthetic pathway	Decrease in phytic acid content, increase in iron and zinc accumulation in grains	Immature embryo	10.8-12.7	*Agrobacterium*	Biofortification of wheat	2021	[123]
TaASN2	Catalyzes the conversion of aspartate to asparagine	Reduced accumulation of free asparagine in grain	Immature embryo	Not reported	Biolistics	Reduce asparagine synthesis in grain	2021	[124]
TaPINb	major gene controlling grain hardness	Harder grains	Immature embryos	0	*Agrobacterium*	Improve grain quality	2021	[125]
TaWAXY	involved in synthesis of amylose	Lower amylose content		33.3				
TaPPO	Catalyses phenol oxidation	Reduced phenol oxidative activity		0				
TaPSY	Involved in carotenoid pathway	Reduced yellow pigment content		6.3				

2.4 Delivery Techniques

At present, *Agrobacterium*-mediated transformation and particle bombardment using immature embryos as explants are the foremost methods for introducing CRISPR/Cas9 constructs into wheat [16]. Traditionally, these DNA-based methods have been used to direct CRISPR/Cas9 expression in target cells via a vector containing the CRISPR/Cas9 cassette. Upadhyay *et al*., [91] transformed wheat suspension cells through *Agrobacterium*-mediated transformation to illustrate the capacity to adapt the CRISPR/Cas9 system to target multiple sites simultaneously. The authors constructed four types of expression vectors by cloning sgRNAs and the Cas9 coding sequence in different combinations. The four constructs contained either a sgRNA and the Cas9 alone, or together on a single construct, as well as designing a construct that expresses two sgRNAs and Cas9 together. For each construct, the expression of the CRISPR/Cas9 components were driven by the constitutive CaMVE35S promoter. Using this approach, the authors targeted the *TaPDS* and *TaINOX* genes, encoding for enzymes involved in the carotenoid biosynthetic pathway and the production of cellulose, respectively. They were able to show the successful targeting of both genes, targeting two sites on each gene individually while also designing a construct that targeted a single site on each gene simultaneously. The authors observed a ME of 18-22% and demonstrated that multiple sites can be targeted by CRISPR/Cas9 using a single expression construct in wheat. Furthermore, amongst the editing spectra they obtained a sequence containing a 24 bp deletion in the *TaINOX* gene and thereby showed that a multiplex CRISPR/Cas9 approach can result in large deletions if two target sites are near each other. Sparks *et al*., [124] recently targeted the *TaASN2* gene in bread wheat which is part of a gene family that encodes for a host of asparagine synthases. These asparagine synthases are involved in the synthesis of a group 2a carcinogen, acrylamide, that is found in some cereal products [126]. The researchers designed four targets to target the first exon of *TaASN2* and assembled them into a single polycistronic gene with each sgRNA separated by tRNA sequences. The tRNA sequences allow for the generation of multiple functioning sgRNAs from a single transgene by exploiting the endogenous tRNA-processing system in plants. The multiplex construct was co-bombarded along with a plasmid carrying the Cas9 gene and another plasmid carrying the *bar* selectable marker gene into immature embryos of bread wheat. The immature embryos were regenerated on selection and the researchers produced 77 T0 plants carrying the Cas9 insert. They then selected 14 T0 plants for self-pollination to generate 167 T1 plants which were analysed for editing events and found 107 edited wheat plants. Downstream analysis of edited T2 plants showed a 90% reduction in asparagine concentration in one edited T2 plant.

These methods result in the integration of CRISPR/Cas9 DNA into host genomes and need to be crossed out to achieve genome-edited plants that do not contain foreign DNA sequences. Furthermore, due to the constitutive expression of the integrated CRISPR/Cas9, unwanted off-target mutations could occur. Additionally, partial CRISPR/Cas9 DNA fragments could potentially be randomly introduced into the plant genome, consequently rendering edited plants prone to regulatory concerns due to the possible presence of foreign DNA sequences [127]. Transgene-free editing

techniques involving transient expression of CRISPR/Cas9 DNA or *in vitro* transcripts (IVTs) in wheat have recently been developed [17]. While plant genome engineering using CRISPR ribonucleoproteins (RNPs) has emerged as an appealing approach that mitigates many of the problems of traditional transformation methods. CRISPR/Cas9 RNPs have been used as a DNA-free strategy to generate precise genomic mutations in numerous plant species including wheat with minimal off-target effects [18].

2.4.1 Transient Expression of CRISPR/Cas9 DNA

In 2016, Zhang *et al*., [17] reported two genome-editing procedures in which mutant wheat plants were regenerated from callus cells that transiently expressed CRISPR/Cas9 components introduced through biolistic bombardment as DNA or RNA. The Transient Expression of CRISPR/Cas9 DNA (TECCDNA) and the Transient Expression of CRISPR/Cas9 RNA (TECCRNA) methods were described as highly efficient techniques to produce non-transgenic wheat mutants.

TECCDNA involves the introduction of plasmid DNA encoding for the CRISPR/Cas9 components that, upon delivery into the target tissue, need only be expressed transiently to introduce mutations to the host genome. Furthermore, since no transgenes are introduced, edited plants are regenerated on media without selection or herbicide.

The aforementioned authors demonstrated the TECCDNA method by targeting the *TaGASR7* gene. The group combined a single sgRNA expression cassette with that of Cas9 on a single DNA construct and introduced the plasmid into embryos of the wheat cultivars Bobwhite and Kenong199. Embryogenic calli were developed in the absence of herbicides or antibiotics on a selection-free medium. They reported MEs of 5.0% and 2.6% in Bobwhite and Kenong199, respectively. They also noted that the efficiency achieved in Bobwhite was higher than that usually found using traditional transformation methods relying on the stable integration of plasmid DNA. Furthermore, among the 80 Bobwhite mutants they identified 51 T0 plants that contained targeted indels in all three genomes. Of these 51 mutants, eight had knockouts across all six alleles. Following these results, the group validated the use of the TECCDNA method by applying it to four other wheat genes. They developed CRISPR/Cas9 DNA constructs for each of the genes, *TaDEP1*, *TaNAC2*, *TaPIN1*, and *TaLOX2* and bombarded them into immature embryos of Kenong199 achieving MEs from 1% to 9.5%. In a later study, the same researchers bombarded 640 immature embryos of Kenong199 with a CRSIPR/Cas9 construct targeting the *TaGW2* gene. Of the 640 bombarded embryos, 30 *tagw2* mutants were obtained for a ME of 4.7% [128].

2.4.2 Transient Expression of CRISPR/Cas9 RNA

Developed in conjunction with the TECCDNA technique, the TECCRNA method functions by transiently expressing *in vitro* transcripts (IVTs) of the Cas9-coding and sgRNA sequences in wheat callus cells. Zhang *et al*., [17] became the first to produce genome-edited plants using CRISPR/Cas9 IVTs. Using particle bombardment, they delivered *in vitro*-transcribed Cas9 mRNA and sgRNA IVTs

targeting the *TaGW2* gene into immature embryos of Kenong199, and plants were regenerated without the use of selective agents. Of the 1600 bombarded immature embryos, they identified 17 T0 mutants (ME 1.1%), while six of those were present with indels in all six *TaGW2* alleles. Notably, the efficiency of this technique is lower than that achieved with TECCDNA which the authors state could be due to the relative instability of RNA compared to DNA. However, TECCRNA is DNA-free and since RNA molecules are highly unlikely to become integrated into the nuclear DNA of plant cells, the mutants produced through this technique are likely to be free of exogenous nucleic acid.

2.4.3 Viral vector-mediated delivery

A major challenge involved in obtaining CRISPR/Cas9 edited plants when using *Agrobacterium*-mediated transformation or a particle gun is that recipient explants need to be regenerated after the GE reagents have been delivered. A promising alternative to the conventional approaches used to deliver CRISPR/Cas9 reagents in plants has emerged in recent years. Virus-based vectors have been touted as a potentially efficient delivery system, particularly for sgRNAs. Virus-mediated delivery strategies afford several advantages over conventional delivery methods; 1) sgRNAs can be attained at high levels due to viral replication occurring autonomously and the viral particles spreading systemically, which could result in a higher editing efficiency, 2) bypasses laborious and time-consuming transformation and tissue culture procedures, 3) can facilitate a multiplex GE approach since multiple sgRNAs can be expressed on a single viral genome, and 4) can be used to develop transgene-free mutant plants [129–131]. Several plant RNA and DNA viruses have been fashioned for the purpose of genome editing in plants. RNA viruses including tobacco mosaic virus (TMV) [132], beet necrotic yellow vein virus (BNYVV) [133], tobacco rattle virus (TRV) [131], and pea early browning virus (PEBV) [134] have been utilised as sgRNA delivery vectors in *Nicotiana benthamiana* and *Arabidopsis thaliana*. Furthermore, in maize and wheat, the RNA virus barley stripe mosaic virus (BSMV) has been employed to deliver CRISPR/Cas9 components [130]. The geminivirus cabbage leaf curl (CaLCuV) has been exploited to deliver sgRNAs in *N. benthamiana* [135]. Geminivirus viral replicons derived from tomato leaf curl virus (ToLCV), bean yellow dwarf virus (BeYDV), and wheat dwarf virus (WDV) have been engineered for GE in tomato [136], potato [137], rice [138], and wheat [139]. However, the lack of viral movement elements in DNA replicons prohibits their spread. Therefore, plant tissues still require *Agrobacterium*-mediated transformation or bombardment of the replicons. Contrary to DNA replicons, RNA virus-derived vectors can be introduced through agroinfiltration. To date, most publications have reported introducing virus-derived vectors that express sgRNAs into plants that are overexpressing Cas9. Upon entry into plant cells, viral genes are expressed through the establishment of the viral expression system. The infected cells will function as a source for the replication of viral genes and the systemic spread into different tissues including germline cells [129,134,140]. This strategy can be used to produce transgene-free mutant plants, especially when using RNA viruses as they do not integrate into the host genome [129,139]. However, RNA viruses are limited by their cargo capacity. Thus, they are unable to carry the large *SpCas9* gene efficiently and are chiefly used to deliver the relatively short

sequences of sgRNAs to Cas9-overproducing plants (Virus-induced plant genome editing). DNA viruses like geminiviruses, on the other hand, have the capacity to carry the large coding sequence of Cas9 but are constrained by limited cell-to-cell movement [141,142].

Due to host range restrictions, RNA virus-mediated genome editing applications have been limited in monocots, especially in hexaploid wheat. Hu *et al*., [130] manipulated a barley stripe mosaic virus (BSMV)-based sgRNA delivery system for CRISPR/Cas9-mediated mutagenesis in wheat. The researchers delivered the BSMV-based vector carrying a single sgRNA targeting the *TaGASR7* gene into wheat plants that were previously transformed with the Cas9 endonuclease gene. They reported targeted mutagenesis at the *TaGASR7* site with efficiencies of up to 78%. Very recently, Li *et al*., [143] also exploited a BSMV-based sgRNA vector to edit different Cas9-transgenic wheat varieties while circumventing the requirement of tissue culture and plant regeneration. The viral vectors were engineered to promote movement of sgRNA transcripts into the shoot apical meristems by fusing mobile RNA elements to the 3' end of the sgRNA, which could facilitate RNA entry into the meristems [144]. Using this strategy, the authors were able to obtain wheat seedlings that contained the desired mutations in up to 100% of the T1 generation. Furthermore, the mutant wheat seedlings were transgene-free with 53.8%-100% of the M1 mutants being virus free.

2.4.4 Ribonucleoproteins

The last few years have seen genome-editing methods being more refined. In response to regulations and public concerns, substantial effort has been directed at developing efficient DNA-free methods of harnessing CRISPR/Cas9 on crop plants. DNA-free genome editing represents a promising tool for plant genome engineering that avoids transgene integration. A ribonucleoprotein (RNP) comprised of a Cas9 endonuclease and a sgRNA can be complexed and used to induce targeted mutations without the need for DNA templates. An RNP is assembled *in vitro* and subsequently introduced into the desired explant material and since the RNP lacks any DNA, the integration of transgenes can be avoided. Woo *et al*., [127] were the first to show that preassembled CRISPR/Cas9 RNPs could be delivered into plant protoplasts of *Arabidopsis*, lettuce, tobacco, and rice. They were able to achieve editing efficiencies ranging from 8.4% to 44% and further demonstrated that the use of CRISPR/Cas9 RNPs fully avoided transgene integration, and further reduced off-target mutations. Since its establishment as a viable method of genome-editing in protoplasts, RNP-mediated genome-editing in protoplasts has been successfully applied in apple and grapevine [145], cabbage [146], and wheat [18]. In plants that are readily regenerated from protoplasts such as lettuce, researchers were able to achieve a ME of 46% in microcalli regenerated from lettuce protoplasts [127]. While recently, Gonzalez *et al*., [147] achieved an editing efficiency of 68% in potato plants regenerated from protoplasts. Alternatively, RNP-mediated genome-editing has been accomplished using particle bombardment on immature embryos of rice [148], maize [149], and wheat [18]. In rice, Banakar *et al*., [148] recently introduced RNPs targeting the *phytoene desaturase* (PDS) gene into scutellum-derived embryos by co-bombarding the RNPs with a plasmid

encoding for *hygromycin phospotransferase (hpt)*. Although this does not strictly fall under DNA-free genome-editing, they achieved a ME of 3.6%. Furthermore, they were able to dramatically improve on this efficiency when using this co-bombardment strategy with an *hpt*-encoding plasmid when the Cas9 protein was mixed with two sgRNAs prior to bombardment. Using this approach, they reported an editing efficiency of 62.9% [150]. Another interesting example of genome-editing by CRISPR/Cas9 RNPs was recently demonstrated in rice. Toda *et al*., [151] delivered RNPs directly into rice zygotes that were produced through *in vitro* fertilisation of isolated gametes. In the absence of selection, the zygotes were regenerated into rice plants that were present with targeted mutations in up to 64% of plants. In maize, Svitashev *et al*., [149] reported MEs ranging from 2.4% to 9.7% in regenerated plants when bombarding immature embryos without selection, and 47% when co-bombarded with a selection marker.

A recent landmark study demonstrated DNA-free CRISPR/Cas9 editing in wheat using RNPs. Liang *et al*. [18] designed RNPs that specifically target the *TaGW2* gene. The guide sequence termed gw2-sgRNA matched the recognition site on homeologs *TaGW2-B1* and *TaGW2-D1* but contained a single mismatch on *TaGW2-A1*. The gw2-sgRNA was complexed to Cas9 and introduced into protoplasts and achieved on-target MEs of 33.4% and 21.8%, for the targets on B1 and D1, respectively. Interestingly, the mismatched target sequence in *TaGW2-A1* contained an off-target editing frequency of 5.7%. By selecting a target site that is very, but not exactly, similar across all homeologs, the researchers were able to formulate a convenient method of comparing on- and off-target editing frequencies. In the same report, they further validated the technique in wheat protoplasts by targeting *TaGASR7* and achieved an efficiency of 45.3%. Once the targets were validated in protoplasts, they delivered the CRISPR/Cas9 components into immature wheat embryos by bombarding them with RNP complexes that were pre-assembled *in vitro.* After bombardment, wheat plants were regenerated without the use of any selectable markers. The researchers successfully produced 28 *gw2* mutant plants of the cultivar Kenong199 from a total 640 bombarded embryos (ME 4.4%) within 7-9 weeks. Of these 28 mutants, 14 were present with indels in *TaGW2-B1* and all 28 in *TaGW2-D1*. Notably, no indels were detected in *TaGW2-A1*. They further reported a ME of 1.8% for immature embryos of the cultivar YZ814 bombarded with the sgRNA targeting *TaGASR7*. The authors demonstrated that RNP-mediated editing of wheat is an efficient and accurate procedure. Deep-sequencing analysis also revealed reduced off-target effects using RNPs compared to DNA-based editing techniques since the delivered components are subject to degradation in the cell and not expressed constitutively. As a result, DNA-free genome editing by RNPs has the potential to alleviate consumer concerns and to bypass regulations by producing transgene-free mutants.

2.4.5 PEG and protoplasts

The methods used to introduce CRISPR/Cas9 constructs into wheat cells were, up until the development of DNA-free techniques, the same as those traditionally used for transgene delivery. In

the past, much energy was focused on developing transient transformation protocols utilising wheat protoplasts since PEG-transformation of protoplasts is very efficient. However, plant regeneration from protoplasts is presently unfeasible in most species. Furthermore, methods for plant regeneration from wheat protoplasts have not yet been developed. Alternatively, PEG-transformation of protoplasts have primarily been used to rapidly validate CRISPR/Cas9 components in transient assays. This technique permits plasmid DNA, *in vitro* transcribed RNA, *in vitro* translated Cas, or whole RNP complexes to be introduced into protoplasts. The activity of the introduced components can then be readily analysed through Sanger or deep sequencing or using the T7E1 assay. Protoplast transient assays have been well exemplified in wheat and provide an efficient method to assess sgRNA editing efficiencies [16,18,139,152,153]. A comprehensive protocol for CRISPR/Cas-mediated mutagenesis in wheat protoplasts has been published detailing target sequence selection, construction, and sgRNA verification [16].

2.5 Mutation detection and analysis

CRISPR/Cas9 genome editing systems can elicit frameshift mutations to knock out target genes. This is done predominantly through the introduction of indels at the target site. The reliable detection of indels is paramount to any CRISPR/Cas9 application [154]. Furthermore, determining whether introduced indels result in frameshifts requires resolution to a single base. Several methods have been developed for the detection of indels. This section will highlight the methods commonly used in literature.

2.5.1 PCR/Restriction enzyme assay

PCR/Restriction enzyme (PCR/RE) assays are commonly used to detect indels in edited cells. The PCR/RE approach uses a restriction enzyme (RE) that contains a recognition site that spans the predicted cut site of the chosen sgRNA. Firstly, the region containing the target site is amplified through PCR. The amplicon is then incubated with an appropriate RE and the digested bands are simply visualised by gel electrophoresis. Digested amplicons represent the wildtype since the native sequence is unchanged. However, if the recognition site is mutated through NHEJ, the enzyme will not cut it and the amplicon would be visible as a single undigested band. PCR/RE assays are simple and straightforward to perform but do not provide any information on the nature of the editing event or whether the indel results in frameshifting or knockout mutations. Therefore, the PCR/RE assay is restricted to use as an initial screening step followed by Sanger sequencing to characterise mutations. Furthermore, the assay is contingent on the target sequence containing a diagnostic recognition site at or near the predicted cut site that will be abolished upon the introduction of an indel. PCR/RE assays are still commonly used since they provide a rapid and cost-effective indication of indel outcomes in genome editing experiments.

2.5.2 T7 Endonuclease I assay

The T7 Endonuclease I (T7EI) assay is another widely used method for the detection of indels generated by genome editing events. This assay involves four steps: 1) amplification of the region

containing the target site, 2) denaturation and reannealing of the dsDNA amplicons to form heteroduplex amplicons with single-stranded 'bubbles' at the mismatch position, 3) treatment of the heteroduplexes with the T7 endonuclease that cleaves double-stranded DNA at the site of the heteroduplex, and 4) detection of the cleavage event through the separation of digested bands by gel electrophoresis. T7EI assays are also simple to perform and are thus commonly used. Similar to PCR/RE assays, they do not inform one on the nature of the edit detected. Furthermore, a common drawback of the T7EI assay is it often does not detect single-base loops resulting from indels [155,156].

2.5.3 Indel detection by Sanger sequence analysis

An alternative to NGS involves the direct Sanger sequencing of amplicons generated from edited samples followed by deconvolution of the resulting traces by online software tools to determine indel frequencies and sizes. The analyses require only two amplicons covering the target site for the edited sample and the wildtype sample, respectively. Amplicons are subsequently Sanger sequenced and the sequencing data file along with the sgRNA sequence are submitted to the software. The software compares the trace of the wildtype to the mixture of traces derived from the edited samples to compute indel profiles. The first of such software to analyse indels elicited by CRISPR/Cas9 was Tracking of Indels by Decomposition (TIDE) [157]. TIDE is a free online software (https: //tide.deskgen.com/) and has become widely adopted. TIDE provides a comprehensive output on indel profiles that is easy to interpret. Indel profiles are projected in a bar graph that shows the size and frequency of individual indels. TIDE can analyse indels in the range of -10 to +10 bp at a single target site and provide the relative frequencies of each type of indel. Recently, a similar software, Inference of CRISPR Edits (ICE) was developed that is also freely available online and straightforward to use (https://ice.synthego.com) [158]. ICE extends on the Sanger sequence trace decomposition method developed by TIDE to include additional features such as displaying the sequence traces of both the wildtype and edited samples to facilitate quality control and visualisation of the edits. The output also includes a list of contributing nucleotide sequences at the site of the indels, however insertions are denoted as 'N'. The range of indels analysed at a single target site is -30 to +14 pb. Moreover, ICE can determine the outcomes of complex editing procedures utilising up to three sgRNAs that target different sites on the same provided amplicon sequence. Through this feature, indels ranging from 100-150 bp or larger can be analysed. ICE outputs also provide the estimated total percentage of each type of indel and the total percentage of indels that result in a frameshift or knockout mutation. The low cost and ease of use of these online tools have made them among the most popular indel profiling methods. Furthermore, recent studies have shown that TIDE or ICE analyses provide editing profiles comparable to NGS assays regarding indel sizes and frequencies [155,158]. Therefore, TIDE and ICE can determine indel profiles in complex sequence spectra to a resolution of a single nucleotide. These methods have been shown to yield almost identical indel profile outcomes in replicate experiments and are able to detect indels present in a sample at frequencies as low as 2% [157,158].

2.5.4 Next-generation sequencing

Targeted Next-generation sequencing (NGS) or amplicon deep sequencing has become a widely adopted method of indel detection in genome editing experiments, as it provides the most extensive indel profiling currently available. NGS approaches are superior to other indel identification strategies with regards to volume of information generated in a single run, as well as sensitivity and accuracy [159]. NGS procedures involve parallel sequencing of a massive number of amplicons derived from the sequence covering the target site. Amplicons are then analysed bioinformatically to determine the indel size distributions and frequencies. Owing to the large number of reads (sequences) generated for each target site, indel profiles can be quantified with very high accuracy [155]. However, the labour, time, cost, and specialist bioinformatic skill requirements needed to apply NGS limits its widespread adoption.

2.5.5 Off-target editing analysis

CRISPR/Cas9 specificity is determined through the complement of the sgRNA sequence and the DNA target sequence. Perfect sequence complementation between the last 8-12 bases of the sgRNA , known as the 'seed sequence', and the equivalent region in the DNA target sequence is critical for effective cleavage by Cas9 [85], however mismatches in sequence complementarity in the PAM-distal region are tolerated [160]. The potential to produce undesired mutations, or off-target effects, in regions of the genome not targeted by the designed sgRNA is an important consideration in the development of CRISPR/Cas9 systems in plants. These concerns can largely be offset by careful sgRNA design [91,161]. The online sgRNA design tools, CRISPR-P [162] and WheatCRISPR [163], implicitly consider off-target effects in the prediction of suitable sgRNAs. However, information on the actual off-target regions is not provided. Different online tools have been developed to predict possible off-target sites and effects, such as CasOT [164]. CasOT is a Perl script used to search genome-wide potential off-targets in a provided genome database. For wheat, this can be retrieved from IWGSC (https://urgi.versailles.inra.fr/download/iwgsc/). Another tool that can be used for screening possible off-target regions in wheat is Cas-OFFinder [165], which also allows for the identification of off-targets specific to various Cas9 orthologues. However, the mentioned strategies only alert the user to potential off-target sites. Indeed, when addressing concerns over off-target effects in plant genomes, emphasis is placed on prevention then followed by detection. The simplest method of detecting off-target events is through PCR, in which flanking regions of potential off-target sites are amplified and Sanger sequenced to determine whether off-target activity occurred [16,166]. This approach, however, suffers the drawback of potentially overlooking mutations at other loci in the plant genome [167]. Alternatively, a commonly used off-target detection method in plants is whole genome sequencing (WGS) which allows for a broad identification of off-target effects. Notably, a recent review found that only one from nine studies detected off-target events using WGS, while the other eight studies found no off-target editing [168]. However, WGS is costly and requires bioinformatic expertise, furthermore, it is unfeasible in hexaploid wheat. Currently, there are no

detection methods that can effectively and economically screen all potential off-target sites [169]. Therefore, careful consideration should be applied to sgRNA design.

2.6 Conclusion

Wheat is one of the most important food crops consumed by humans. It provides approximately 20% of the world's total calories [170]. While much has been achieved in wheat yield gains since the 1970s, the global average wheat yield currently stands at approximately 3 tons per hectare [171]. Furthermore, wheat yields need to be increased by an estimated 60% by 2050 to feed a projected world population of 9 billion people [171]. To confront this challenge and break through the current yield ceiling, improved genetic engineering and molecular-based breeding techniques will be a necessity. The challenge is further exacerbated by current regulations on transgene products. The development of DNA-free genome editing techniques, such as the biolistic delivery of CRISPR/Cas9 components through RNP complexes, provide an efficient means to genetically improve bread wheat while mitigating impeding regulatory concerns.

Chapter 3: Genome editing in wheat using CRISPR/Cas9 ribonucleoproteins

3.1 Introduction

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) was first adapted from its native function in bacterial adaptive immune responses to target eukaryotic genes in 2012 [16]. Presently, the most popular system used in plants involves the Cas9 endonuclease complexed to an artificially merged single guide RNA (sgRNA) containing a target-specific spacer sequence of 20 nucleotides. The CRISPR/Cas9 system can theoretically be designed to target any genomic sequence provided the selected sequence meet two criteria: first, the target sequence is unique, and second, the target sequence is directly adjacent to the Protospacer Adjacent motif (PAM) represented by the sequence NGG [172,173]. Since its establishment, the CRISPR/Cas9 system has been used to generate targeted genomic modifications in numerous crops [145,174–176]. The CRISPR/Cas9 apparatus are typically delivered into cells through plasmids introduced via either *Agrobacterium* or particle bombardment. This regularly results in the intentional (and unintentional) incorporation of segments of the DNA construct into the target plant's genome, consequently producing transgenic organisms [139]. Furthermore, the stable integration of plasmid DNA expressing CRISPR/Cas9 components could significantly increase the likelihood of off-target effects. Currently, the removal of transgenes is done through crossing transformed plants with untransformed plants [177]. This, however, is challenging in certain plant species and could potentially require multiple generations to achieve, further increasing the labour and time required to incorporate a desired trait into a plant. Moreover, transgenic plants and undesired off-target mutations are likely vulnerable to regulatory and public concerns [178]. Accordingly, much effort has been directed at finding transient transformation strategies that address these problems. A recent advancement in transient transformation using CRISPR/Cas9 ribonucleoproteins (RNPs) has been published. Since RNPs are DNA-free, they avoid integrating into host genomes. Furthermore, they have been found to act almost instantly after being introduced and are subsequently rapidly degraded [147], therefore reducing the likelihood of off-target effects significantly. Furthermore, RNPs are simple to produce as they bypass the need of creating plasmids while also being more cost efficient relative to some other transformation procedures [18] as they don't require selective tissue culture. Liang *et al*., [18] reported the use of transcribed sgRNAs complexed to Cas9 *in vitro* to produce edited wheat plants. They used particle bombardment to introduce RNPs into immature wheat embryos, which were then regenerated on selection-free media. Crucially, the authors reported that no off-target mutations were detected and further concluded that the mutant plants were free of any exogenous DNA.

Wheat, as one of the world's most important crops, has received much attention following the advent of CRISPR/Cas9 technology to study gene function and improve agronomic traits [172,179–181]. CRISPR/Cas9 is proposed as having the potential to substantially facilitate crop breeding, especially

in wheat. Furthermore, the rapid production of wheat mutant plants that do not require laborious and time-consuming crossing procedures aimed at producing transgene-free mutants will further expedite research in this important crop. For CRISPR/Cas9 technology to meet its potential requires the wide-scale adoption and validation of this technique. Hence, in this study we aimed to establish the use of CRISPR/Cas9 RNPs to produce edited wheat plants. sgRNAs were designed to target the *TaLCYB* gene, which plays a central role in the β-carotene biosynthetic pathway in wheat [182]. The successful knocking out of *TaLCYB* could result in a detectable bleached phenotype which could aid in the screening of mutant wheat plants.

3.2 Materials and Methods

3.2.1 Design of sgRNA targets

Three sgRNAs targeting the lycopene β-cyclase *(LCYB*) gene in *Triticum aestivum* were selected using the online wheatCRISPR software (https:// crispr.bioinfo.nrc.ca/WheatCrispr/). Target candidates were selected based on the overall on-target and off-target scores returned. Top scoring potential guide sequences were chosen according to whether they target regions that are conserved across all three genomes and if they were present with a restriction enzyme recognition site spanning the predicted Cas9 cut site 3-5 base pairs upstream of the PAM. The selected targets were then blasted against the IWGSC RefSeq v1.0 to determine possible off-target complementarity.

3.2.2 Generation of sgRNA cassettes

To construct the sgRNA intermediate vector, specific primers were designed to amplify the gRNA (guide RNA) scaffold sequence from the pDIRECT_22C template plasmid (Addgene plasmid # 91135 ; http://n2t.net/addgene:91135 ; RRID:Addgene_91135). The forward primer contained 22 bases on the 3'-end that were complimentary to the gRNA scaffold sequence, followed by a 25 bp non-complimentary overhang designed with two *Bbs*I recognition sites and a *Aat*II recognition site at the 5'-end (Figure 3.1 and primer sequences shown in Table 3.1). The reverse primer contained 18 bases on the 3'-end complimentary to the 3'-end of the gRNA scaffold sequence and contained a 5'-overhang containing a *Spe*I recognition site. The primers were used to amplify the gRNA scaffold. The 110 bp amplicon containing the gRNA scaffold and the pGEM-T-easy vector (Promega) were digested with *Aat*II and *Spe*I to produce complimentary overhangs. The gRNA scaffold amplicon was ligated into pGEM-T-easy to produce the pGEM-Scaffold vector.

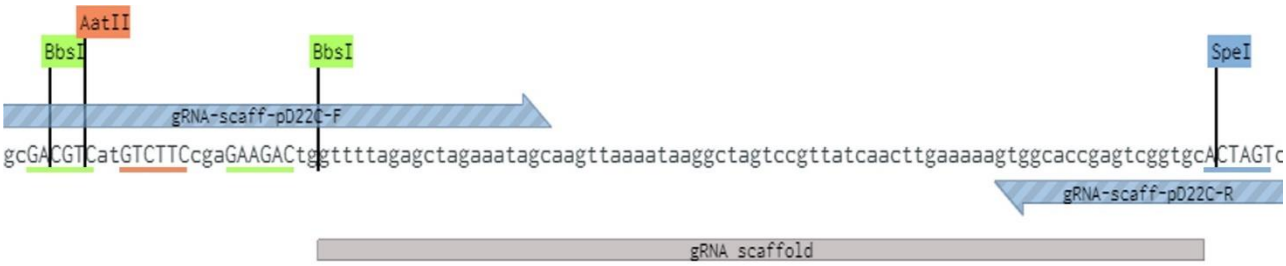


Figure 3.1. Amplicon containing the gRNA scaffold amplified from pDIRECT_22C. Forward and reverse primers are blue. The gRNA scaffold on the template spans the grey bar. Primers were designed with overhangs containing RE recognition sites. AatII (recognition site underlined in orange) and SpeI (recognition site underlined in blue) digestion of the resulting amplicon provides overhangs for insertion into pGEM-T-easy. BbsI (recognition sites underlined in green) digestion of the pGEM-T-easy vector containing the gRNA scaffold insert provided the site for the insert of sgRNAs.

Table 3.1 Primer sequences used for the amplification of the gRNA scaffold from the pDIRECT_22C plasmid

Primer Name	Sequence (5'-3')	Amplicon length (bp)	Function
gRNA-scaff-pD22C-F	gcgacgtcatgtcttccgagaagactggttttagagctagaaatagc	110	Amplify the gRNA scaffold from the pDIRECT_22C plasmid and introduce a *Aat*II cut site for cloning into pGEM-T-Easy. *Bbs*I cut sites are added for insertion of sgRNA sequence.
gRNA-scaff-pD22C-R	gactagtgcaccgactcggtgccac		Amplify the gRNA scaffold from the pDIRECT_22C plasmid and introduce a *Spe*I cut site for cloning into pGEM-T-Easy.

To construct the sgRNA expression vector, the target sequences of 20 bp were ordered as synthesised oligonucleotides from Integrated DNA Technology (IDT, https://www.idtdna.com). The synthesised guide sequences had CGTC added to their 5' ends for the forward strand and AAAC added to the 5' ends of the reverse strand. These two tails function as 5' overhangs in the cloning reaction. The guide sequence oligos were annealed by combining 1.5 µl of each oligo (100 µM) with 1X NEBuffer 2.1 in a total reaction volume of 50 µl. The reaction was heated to 95°C for 4 min followed by 70°C for 10 min and then left to cool to room temperature.

The annealed oligos were inserted into pGEM-scaffold by assembling 1 µl annealed oligos, 20 ng of *Bbs*I-digested pG-T7-scaf, 1X T4 DNA ligase buffer, and 1 unit T4 DNA ligase (Promega, USA) in a total reaction volume of 20 µl. The reaction was left at 4°C for 16 hrs.

3.2.3 Generation of sgRNA transcripts

To produce the sgRNA transcripts that would be complexed to the Cas9 endonuclease, the pGEM-scaffold vector containing the guide insert was digested using the restriction enzyme *EcoR*I as follows: 1X CutSmart buffer, 1 µl *EcoR*I, and 2 µg of pGEM-sgRNA in a total reaction volume of 50 µl. The linearised pGEM-sgRNA was isolated and sgRNA transcripts were synthesised using the

HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England biolabs, E2050S). The sgRNA transcripts were subsequently purified using the Zymo RNA clean and concentrator kit (Zymo Research, USA). Aliquots of 10 µl were made at a concentration of 1µg/µl and stored at -80°C.

3.2.4 *In vitro* assessment of sgRNAs

To assess the guide specificity and the Cas9 nuclease activity on the target site, an *in vitro* assessment of the RNP complexes were performed for each target sequence. The target DNA template was produced by amplifying a fragment of the gene that contains the sgRNA targeting sequence using the High-Fidelity Phusion polymerase (New England Biolabs, USA). The PCR product was validated by Sanger sequencing. Cleavage reactions were set up as follows: 1X Cas9 reaction buffer, 1 µg Cas9 protein (TrueCut Cas9 Protein v2; Thermo Scientific, USA), 1 µg sgRNA, and 100 ng PCR amplicon in a reaction volume of 20 µl. The reactions were incubated at 37°C for 1 hr followed by 65°C for 10 min to deactivate the Cas9 nuclease. Finally, the products were run on a 1% agarose gel to evaluate cleavage efficacy.

3.2.5 Biolistic bombardment of RNPs

3.2.5.1 Preparation of immature embryos

Wheat spikes of the cultivar Bobwhite were collected approximately 14 days after pollination (DAP), when the immature embryos were 1-1.5 mm in diameter. The immature grains were separated from the spikes and placed in a sterile 50 ml falcon tube. The grains were then sterilised in a laminar flow cabinet using 70% EtOH for 2 min followed by washing with 2% sodium hypochlorite for 20 min. The grains were then rinsed three times with sterile distilled water. Immature embryos were excised from the grains using fine forceps under an optical microscope. Excised immature embryos were placed scutellum side up in the central region of osmotic media (OSM) (Murashige and Skoog (MS) (Duchefa M0222): 4.33 g/L, Mannitol: 72.9 g/L, 2,4-D: 5 mg/L, Phytagel: 3.2 g/L, pH: 5.8) plates. Approximately 80 embryos were deposited per plate. The plates were incubated overnight in the dak at 24°C.

3.2.5.2 Preparation of gold particles

The gold microcarriers were sterilised by adding 1 ml 100% ethanol to 40 mg 0.6 µm gold (BioRad, USA) particles in a 1.5 ml Eppendorf tube and vortexed for 2 min. The gold particles were left to soak for 15 minutes then the mixture was centrifuged at 13 000 rpm for 1 min. The supernatant was removed, and the gold particles were washed by adding 1 ml sterile water then vortexing thoroughly and pelleting the gold particles by centrifugation then removing the supernatant. The wash step was repeated 3 times. Finally, the gold was resuspended in 1 ml sterile RNase-free water at a concentration of 40 mg/ml. Aliquots of 50 µl were prepared and stored at -20°C.

3.2.5.3 Coating of gold particles with RNPs

To coat the CRISPR/Cas9 RNPs onto the gold nanoparticles, the CRISPR/Cas9 components were assembled as follows (sufficient for 10 shots): 20 µg Cas9 protein, 20 µg sgRNA, 10 µl 10X Cas9

reaction buffer, filled to a total reaction volume of 100 µL with RNase-free water. The 10X Cas9 reaction buffer was made as follows: 2 mL of 1M HEPES, 1 mL of 1M $MgCl_2$, 50 µL of 1M DDT, 5 mL of 3M KCl, filled up to a total volume of 10 mL with 1.95 mL of RNase-free water. The CRISPR/Cas9 reaction mixture was homogenised by gently tapping the tube. The reaction was then incubated at RT for 10 min. Thereafter, 50 µL of gold nanoparticles (prepared in section 3.2.5.2) was added and thoroughly but gently mixed by pipetting.

3.2.5.4 RNP bombardment procedure

To perform the biolistic bombardment with the CRISPR/Cas9 RNPs, 15 µl of the Cas9 RNP-coated gold nanoparticles were deposited and spread across the central region of the macrocarrier and allowed to air-dry for 1 h at RT in the laminar flow hood. The immature embryos on OSM (prepared in section 3.2.5.1) were then bombarded at a target distance of 6.0 cm with a rupture pressure of 1 100 psi using a Bio-Rad PDS-1000 He™ particle gun. Bombarded embryos were kept on osmotic media in the dark at 24°C overnight.

3.2.6 Tissue culture

One day after bombardment, the embryos were transferred to plates containing recovery media (REM) (MS including vitamins (Duchefa M0221): 4.4 g/L, Sucrose: 30 g/L, 2,4-D: 2 mg/L, NZ-Amine A: 0.5 g/L, $CuSO_4.5H_2O$: 0.6 mg/L, Phytagel: 3.2 g/L, pH: 5.8). Thirty embryos were deposited on each plate. The plates were then incubated in the dark at 24°C for 14 days. Healthy calli that had proliferated to a size between roughly 4-6 mm were transferred to regeneration media (RGM) (MS including vitamins: 4.4 g/L, Sucrose: 30 g/L, Kinetin: 0.2 mg/L, Phytagel: 3.2 g/L, pH: 5.8). The calli were then incubated for 14 days at 24°C and exposed to a light-dark cycle of 16h/8h. Using sterile forceps, the calli were separated into 2 to 3 smaller pieces and placed onto fresh regeneration media (RGM) and incubated under the same conditions as before for a further 14 days. Regenerated plantlets with leaves with a length exceeding 1.5 cm were transferred to rooting media (RTM) (MS including vitamins: 2.2g/L, Sucrose: 30 g/L, Kinetin: 0.2 mg/L, NAA: 0.05 mg/L, Phytagel: 3.2 g/L, pH: 5.8) and incubated in the same conditions as previously.

3.2.7 Early assessment of explants

3.2.7.1 PCR/Restriction enzyme assay

Four days after bombardment, approximately 100 embryos were collected at random for genomic DNA extraction using the CTAB method [183]. The target gene was PCR-amplified using High-Fidelity Phusion polymerase (New England Biolabs, USA) with genome-specific primers (primer sequences in Supplementary Table 1). Then the PCR products were separated using electrophoresis on a 1% agarose gel and PCR products corresponding to the correct size were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA). Purified PCR products were subsequently digested with a restriction enzyme containing a recognition site spanning the predicted Cas9 cut site. Digested products were separated on a 1% agarose gel for visualisation.

3.2.7.2 Gene-editing analysis by Sanger sequencing

The digested PCR products (prepared in section 3.2.7.1) were Sanger sequenced at CAF (Stellenbosch University, SA). CRISPR-Cas9 DNA changes were calculated based on the insertions and deletions around the cleavage site (3 bp upstream of the PAM sequence) using the Inference of CRISPR Editing Software- ICE software v.2 (Synthego Corporation, Palo Alto, CA, USA).

3.2.7.3 Mutation characterisation

PCR products (prepared in section 3.2.7.1) were A-tailed by assembling the following reaction: 7 µL of purified PCR product, 0.5 µL dATP (100 µM), 4 µL of 5X GoTaq buffer, 0.2 µL GoTaq DNA polymerase (Promega), and 8.3 µL of PCR-water for a total reaction volume of 20 µL. The reaction was incubated for 10 min at 72°C in the thermocycler. The A-tailed PCR product was then cloned into pGEM-T-easy in a ligation reaction done as follows: 5 µL 2X rapid ligation buffer, 1 µL linearised pGEM-T-easy vector, 3 µL A-tailed PCR product, and 1 µL T4 ligase. The reaction was incubated overnight a 4°C. DH5α *E.coli* was transformed with 5 µl of the ligation reaction and plated on LB agar plates supplemented with 100 mg/L ampicillin, 0.5 mM IPTG, and 80 µg/mL X-gal. Plates were incubated at 37°C for 16 hrs. White colonies were picked from the culture plates and added to 5 ml liquid LB culture containing 100 mg/L ampicillin and incubated for a further 16 hrs at 37°C at 200 rpm. Plasmid DNA was isolated and sent for Sanger sequencing using primers M13F and SP6.

3.3 Results

3.3.1 Selection of LCYB targets

The gene chosen for Cas9-mediated targeting is Lycopene β-cyclase (*TaLCYB)*, an important enzyme involved in the carotenoid biosynthesis pathway in several plant species [184]. The *LCYB* sequence obtained from URGI (https://urgi.versailles.inra.fr/download/iwgsc/) of Chinese Spring was used to design primers (sequences provided in Supplementary Figure 1) to amplify an 812 bp intronless fragment in Bobwhite DNA. Three sgRNAs were designed from the resulting Bobwhite *LCYB* sequence to target three different loci (Figure 3.2). sgRNA sequences and corresponding CRISPR-P (http://crispr.hzau.edu.cn/) and CRISPRwheat (https://crispr.bioinfo.nrc.ca/WheatCrispr/) scores are provided in Table 3.2. The sgRNAs were ultimately selected due to the presence of RE recognition sites that spanned the predicted cut-site of Cas9.

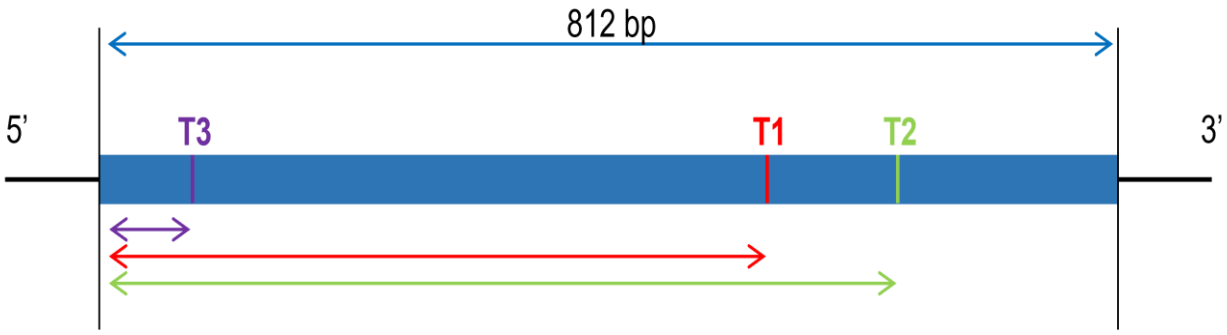


Figure 3.2. Structure of the LCYB amplicon with estimated positions of selected sgRNA targets. Red: LCYB-T1; Green: LCYB-T2; Purple: LCYB-T3.

Table 3.2 Selected sgRNA targets. PAM sites for each target are in brackets. REs: The Restriction enzymes used to screen for sgRNA activity in vivo.

				Score		
Gene	**Target name**	**Target sequence and PAM site (5'-3')**	**REs**	**CRISPR-P**	**WheatCRISPR**	**Targeted Genome copies**
TaLCYB	LCYB-T1	CGACCAGCGACGTCTCCTCG(AGG)	*Psp*XI	0.1041	0.443	A,B,D
	LCYB-T2	GATGCAGCGGTCCATCATGG(TGG)	*Bst*XI	0.0656	0.445	A,B,D
	LCYB-T3	GCGCGCCACCATGTACCCTG(TGG)	*Xcm*I	0.0469	0.495	A,B,D
TaGW2	gw2-sgRNA	CCAGGATGGGGTATTTCTAG(AGG)	*Xba*I	0.0269	0.417	B,D

The gw2-sgRNA sequence was obtained from a paper published by Liang *et al*., [18]. They recommend the targeting of the *TaGW2* gene as a control for verification and optimisation. This gene is involved in grain weight control [185]. Its use allows for the verification of an already published target. Further, since the gw2-sgRNA matches perfectly with the sequences in *TaGW2-B1* and *-D1*, but contains a single mismatch in *TaGW2-A1*, it could allow for the measurement of the target's efficiency between homeolog targets as well as off-target effects.

3.3.2 The construction of sgRNA expression cassettes

Once sgRNAs were selected and synthesised, they were each separately cloned into the pGEM-scaffold vector to produce the expression cassette pGEM-sgRNA (Figure 3.3). The sgRNA sequence was inserted under the T7 promoter for expression and directly upstream to the gRNA scaffold. The pGEM-scaffold vector was designed so that only the target sequence needs to be replaced to target a different genomic site; the gRNA scaffold remains unchanged. For transcription, the pGEM-sgRNA plasmid was linearised by digestion with *Eco*RI and the target-specific sgRNA was transcribed from the T7 promoter. Sequencing results of the target containing pGEM-sgRNA plasmids are provided in Supplementary Figure 2.

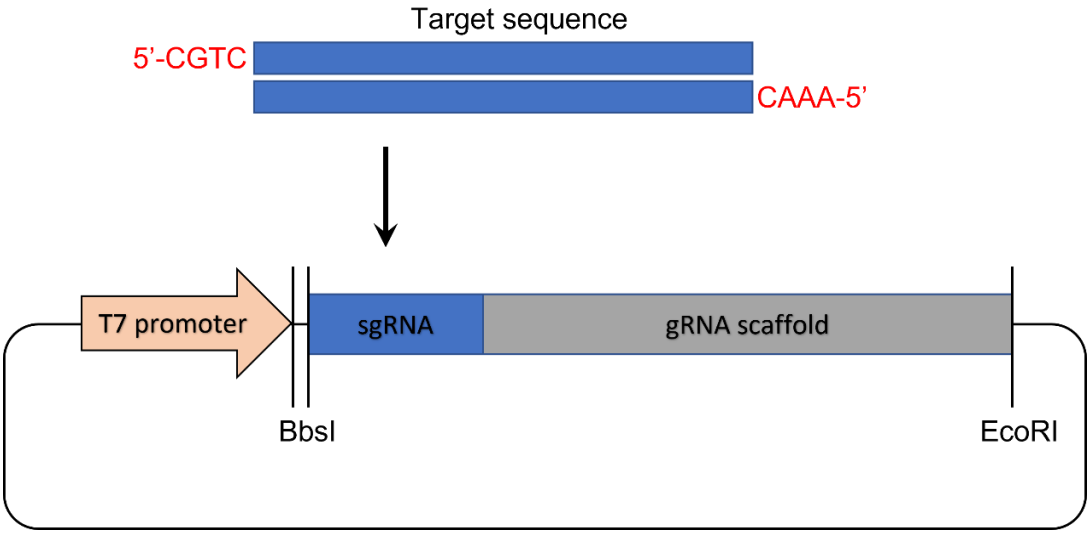


Figure 3.3. Schematic for cloning the guide sequence oligos into the pGEM-scaffold vector containing the gRNA scaffold. The annealed oligos contain overhangs (red letters) for ligation into the pair of BbsI sites in the pGEM-scaffold vector. sgRNAs were transcribed using EcoRI-linearised pGEM-sgRNA.

3.3.3 *In vitro* assessment of sgRNAs

It is recommended that preassembled RNPs be validated prior to its introduction into target tissues. To validate that the sgRNA and Cas9 are complexed *in vitro* and that the targeted RNP cuts at the desired locus, an *in vitro* assay was performed for the targets LCYB-sgRNA-T1, -T2, and -T3. 200 ng of PCR product containing the target sites was used as a template for each reaction. As shown in Figure 3.4, each sgRNA produced smaller fragments after cleavage. The expected band sizes for T1, T2, and T3 were 534 bp and 278 bp, 746 bp and 66 bp, and 640 bp and 170 bp, respectively. The uncut PCR product was used as a control in each assay and is 812 bp in length. Included in the sgRNA-T1 assay in lane 2 is the digestion of the PCR template by the RE *Psp*XI which cuts at the same predicted site as Cas9 for LCYB-sgRNA-T1. The assays of sgRNA-T1 and -T2 also include the reaction containing an amplicon derived from the sequence of Cas13 with Cas9 and the respective guides in lane 3. The Cas13 amplicon does not contain any of the target sequences and this was done to determine whether any indiscriminate cleavage occurs. The three *LCYB* targets were effective at cleaving the target sites and suitable for further use in gene editing.

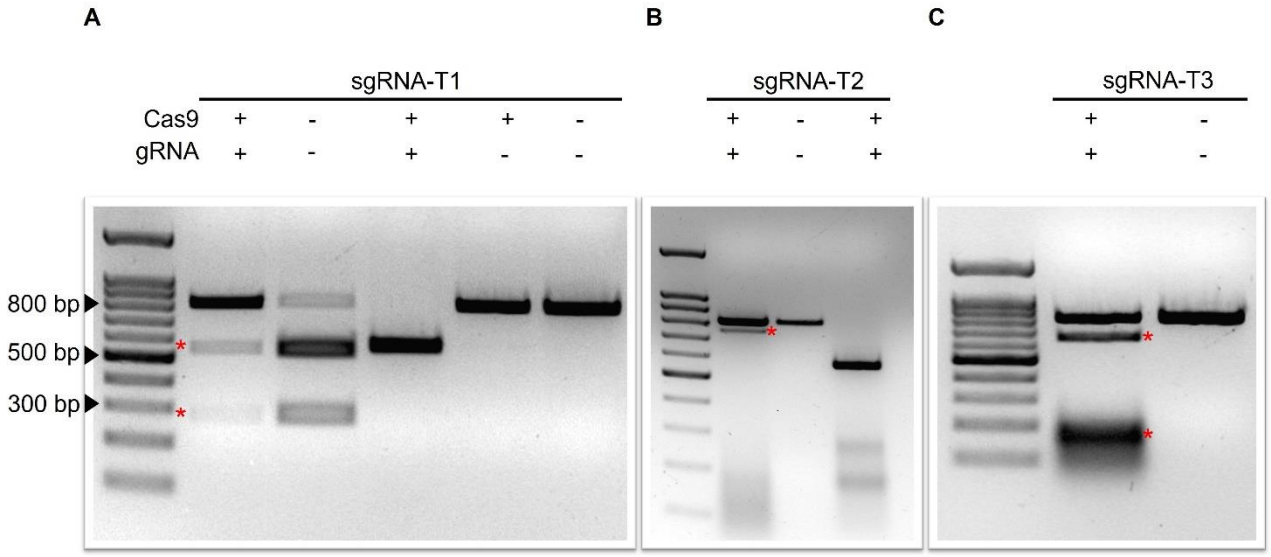


Figure 3.4. In vitro assay of the three sgRNAs targeting TaLCYB. A) lcyb-sgRNA-T1. 1. Cleaved fragments 2. PspXI digestion 3. Cas13 amplicon 4. LCYB target amplicon with Cas9 but without guide 5. LCYB uncleaved control template B) lcyb-sgRNA-T2 1. Cleaved fragments 2. Control template 3. Cas13 amplicon C) lcyb-sgRNA-T3 1. Cleaved fragments 2. Control template. 100 bp ladder used in all three gels. Red asterisks indicate digested fragments.

3.3.4 Biolistic transformation of RNPs

Ribonucleoprotein complexes were transformed into wheat immature embryos of the cultivar Bobwhite through biolistic bombardment as described in a previous study [186]. Following bombardment, embryos were regenerated on selection-free media. The three transformation events are summarised in Table 3.3.

Table 3.3 Summary of transformation events using RNPs.

Event name	Target introduced	Total number of embryos	Regenerants	Screened embryos	Inferred editing by ICE v2
GW2-RNP	sgRNA-GW2	150	23	75	Yes
T1-RNP	sgRNA_T1	390	25	100	No
T2-RNP	sgRNA_T2	150	NA	80	No

3.3.4.1 Early assessment to validate RNP entry

Immature embryos were harvested at random four days after transformation and pooled. DNA was extracted from these pools of immature embryos and the fragments flanking the respective target regions were amplified to evaluate RNP editing in wheat cells. For the transformation event GW2-RNP, the prior validation procedure of an *in vitro* cleavage assay was not performed since it was a target retrieved from literature. The gw2-sgRNA was delivered into 150 embryos in total. Of those, 75 were collected at random four days after bombardment for early analysis. The 75 embryos were separated into pools of 10, except for pool 8 which contained 5 embryos. DNA was extracted from each pool of embryos and the GW2 fragment containing the target region of each homeolog was amplified using genome-specific primer pairs, GW2-A1-F and GW2-A/B/D-R, GW2-B1-F and GW2-

A/B/D-R, and GW2-D1-F and GW2-A/B/D-R, for GW2-A1, -B1, and -D1, respectively. The amplification of the target region using genome-specific primers was done because of the mismatch present in the GW2-A1 target which would contribute to too much noise resulting in an inability to parse possible mutations from the wild type (WT) sequences. The amplicons were subsequently digested with the RE *Xba*I to enrich for possible mutations amongst the WT sequences. *Xba*I has a recognition site spanning the predicted cut site of the gw2-sgRNA guided Cas9, therefore any indels introduced at the cut site would eliminate the *Xba*I recognition site. The *Xba*I-digested amplicons were sanger sequenced and subjected to the online bioinformatic software ICE v2. ICE (inference of CRISPR Edits) is a bioinformatics software tool developed to analyse pooled CRISPR-edited DNA using Sanger sequencing data [158].For the B-amplicon, one of the eight pools were determined to contain edited sequences (Figure 3.5a). The pool, GW2-B2, had a total indel percentage of 3%. The contributing sequences and their frequencies were 96% WT, and 3% of sequences contained a +2 insertion. The GW2-B2 pool had a knockout score of 3. The remaining B-amplicon pools were determined not to be present with indels at the target site with knockout scores of 0 for all. Six of the eight D-amplicon pools were determined by ICE v2 to contain mutated sequences. The sequence analysis of the mutation-containing pools is shown in Figure 3.5b. The total indel percentage ranged from 0% to 14%, while four pools had a total indel percentage of 7% or higher. All edited pools contained the same type of edit, a two-nucleotide deletion following the expected cut site of the sgRNA. The samples GW2-D5 and GW2-D6 exhibited knockout scores of 13 and 14, respectively. These two samples represented the highest scoring pools and were predicted to also contain sequences present with a two-nucleotide deletion immediately before the predicted cut site of the sgRNA. The GW2-D6 sample's breakdown of contributing sequences were 82% WT, 10% containing the 2 bp deletion following the cut site and 4% with a 2 bp deletion before the cut site.

A

GW2-B2	Total indel %: 3	Knockout score: 3
Indel	Contribution	Sequence
0	96%	GGCATTCGTTGCAGCTCCTCCTCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG
+2	3%	GGCATTCGTTGCAGCTCCTCCTCTA|nnGAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTAT
GW2-B5	Total indel %: 0	Knockout score: 0
Indel	Contribution	Sequence
0	100%	GGCATTCGTTGCAGCTCCTCCTCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG

B

GW2-D5	Total indel %: 13	Knockout score: 13
Indel	Contribution	Sequence
0	84%	GGCATTCGTTGCAGCTCCTCCTCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG
-2	7%	GGCATTCGTTGCAGCTCCTCCTCTA|--AATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG
-2	6%	GGCATTCGTTGCAGCTCCTCCTC--|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG
GW2-D6	Total indel %: 14	Knockout score: 14
Indel	Contribution	Sequence
0	82%	GGCATTCGTTGCAGCTCCTCCTCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG
-2	10%	GGCATTCGTTGCAGCTCCTCCTCTA|--AATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG
-2	4%	GGCATTCGTTGCAGCTCCTCCTC--|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGG

Figure 3.5. ICE analysis of CRISPR/Cas9 RNP edited pools. Digested PCR amplicons derived from pools of embryos bombarded with the sgRNA-GW2 target were Sanger sequenced and analysed with the online ICE software. Sequence breakdown of the GW2 PCR amplicons of the A) B genome and B) D genome. 'Total indel %' refers to the percent of the total pool of sequences that are not WT sequences. 'Knockout score' is the proportion of sequences in each sample that have a frameshift mutation. The sgRNA target sequence is shown in red. The vertical red line represents the expected cut site. The underlined sequence is the PAM sequence. 'Indel' is the number of nucleotides inserted or deleted in each sequence. 'Contribution' is the percent of all sequences in the sample present with the type of indel indicated. Indels are represented by ''n'' or ''– ''in green.

To further characterise the predicted indels, the *Xba*I-digested GW2-D amplicons of the pools that were predicted by ICE v2 to harbour mutated sequences were cloned into pGEM-T-easy and sequenced. Figure 3.6 shows four clones that were present with polymorphisms around the cut site.

WT		GCTCCTCCTCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGGCTGAGCACCAGCCATCAAGCATGG
	Nucleotide change	
GW2D-1	A/G	GCTCCTCCTCTA|GGAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGGCTGAGCACCAGCCATCAAGCATGG
GW2D-3	G/T	GCTCCTCCTCTA|TAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGGCTGAGCACCAGCCATCAAGCATGG
GW2D-8	T/C	GCTCCTCCCCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGGCTGAGCACCAGCCATCAAGCATGG
GW2D-19	T/C	GCTCCTCCCCTA|GAAATACCCCATCCTGGTGGATTTTCTTGTGCTGTTGCTGCTATGGCTGAGCACCAGCCATCAAGCATGG

Figure 3.6. Polymorphisms present near the expected cut site in sequences derived from D genome amplicons of pools predicted by ICE to have been edited. 'WT' is the wild-type sequence. GW2D-# refers to a given sequence. 'Nucleotide change' is the base change compared to the WT and is shown in green. The sgRNA target sequence is shown in red. The vertical red line represents the expected cut site. The underlined sequence is the PAM sequence.

Transformation events T1-RNP and T2-RNP were screened as was described for GW2-RNP. One hundred embryos and 80 embryos were screened for T1 and T2-RNP, respectively. However, ICE did not detect any indels in either of these events.

3.4 Discussion

Liang *et al.,* recently used CRISPR/Cas9 RNPs to successfully edit wheat [18,186]. They made use of particle bombardment of immature embryos with sgRNAs that were transcribed *in vitro*. Subsequently, they regenerated plants through callus formation and tissue culture. The authors reported a transformation efficiency of 4.4%, which is comparable to other transformation techniques in wheat. Additionally, they found that mutants were transgene-free, and that no off-target mutations occurred. Therefore, they presented a feasible approach for the successful transformation of wheat through CRISPR/Cas9 that could circumvent some limitations of other DNA-based transformation methods. In this study, we followed the published protocol of Liang *et al.,* for the transformation of wheat immature embryos with RNPs.

The sgRNAs selected for this project were not the highest scoring targets estimated by two sgRNA scoring softwares, CRISPR-P and wheatCRISPR. Instead, considerations regarding restriction enzyme recognition sites spanning the predicted cut site were prioritised. This was done to facilitate a convenient method of screening possible mutants through PCR/restriction enzyme (PCR/RE) assays. Furthermore, the variance in allocated scores between different softwares', as can be seen amongst our selected targets, does not decisively elect a better sgRNA target. Also, the low score returned for a target that has been previously validated and found to be efficient *in vivo* suggests that the scoring results should be interpreted with caution. Liang *et al*., suggests that sgRNA target sites be selected manually based on the criterion that a 5'-NGG PAM is present directly downstream of the target sequence. Furthermore, regardless of predicted target and off-target scores provided by online tools, testing the efficacy of sgRNA targets still depend on empirical results [187]. Designed sgRNAs should be tested *in vitro* prior to its *in vivo* application. In bread wheat, an allohexaploid, genes are mostly present in three copies (A, B, and D). Gene homeologs are often of a redundant nature, compensating for knocked out gene copies by overexpressing in another copy [49,112]. The three sgRNAs selected in this study for targeting the *TaLCYB* gene were designed to target conserved regions on all homeologs. As noted, *LCYB* is part of the carotenoid biosynthesis pathway in several plant species [184]. It has also been implicated as playing a central role in β-carotene biosynthesis in wheat [182]. Additionally, designing sgRNA targets that could mutate regions conserved across all three genomes simultaneously could increase the likelihood of detecting phenotypic effects that can be concealed by redundant non-mutated gene copies. For example, phytoene desaturase (*PDS*), which is also involved in the carotenoid biosynthetic pathway, is commonly used as marker gene for Cas9-induced knockout and gene silencing approaches in many plant species, including wheat [188–190]. A knockout of the gene results in the repression of the biosynthesis of chlorophyll and a subsequent photobleached phenotype. The previously published

gw2-sgRNA contained a single mismatch in the A homeolog. The three *LCYB* targets were all validated and deemed effective through *in vitro* assays. An *in vitro* assay of sgRNA-gw2 was not performed because it had been validated in a previous study [18].

The detection of edited mutants is central to any CRISPR/Cas9 application. This becomes difficult in plants with complex genomes, such as polyploid wheat. A commonly used qualitative method for detecting mutations is the PCR/RE assay. In this method, detection is facilitated by REs that have a recognition site spanning the Cas9 endonuclease cutting site three base-pairs upstream of the PAM. Following editing, the target site is PCR-amplified, and mutations are detected through a restriction enzyme digestion reaction. Theoretically, if the PCR product is completely digested then no mutations are present, while undigested bands would indicate an edited sequence [16]. In this study, PCR/RE assays were used on amplicons generated from template DNA extracted from pools of immature embryos that were bombarded. However, due to the total amount of WT sequences to be expected in a sample containing pooled DNA, it was not informative. Furthermore, the immense number of DNA copies present in a PCR product renders the complete digestion of all WT sequences ineffective. Consequently, the digested amplicons were Sanger sequenced to determine whether editing had occurred. Sanger sequence results are usually presented in chromatograms with the occurrence of nucleotides characterised as coloured peaks. Since a large pool of DNA will contain largely un-mutated DNA as well as a variety of edit types, chromatograms of pooled DNA containing mutated sequences are unlikely to show any noticeable indication of mutation efficiency or the type of mutations present in the pool. Therefore, possible changes were visualised using an online software established to analyse edited pooled DNA using regular Sanger sequence data called ICE (Inference of CRISPR edits) [158]. ICE uses trace data from Sanger sequencing to deduce the presence of indels, and their frequencies. It provides detailed information about the size and types of indels in a given trace sequence [104]. It has been shown recently that the ICE-software correlates strongly with that of next-generation sequencing using Sanger sequencing data and has been used to detect mutations in pooled DNA samples in previous studies [191,192].

The targets LCYB_T1 and LCYB_T2 were bombarded into immature wheat embryos in two separate events. Collectively, 540 embryos were bombarded in total and of those 180 were screened for mutations four days after transformation using the strategy outlined above. For the LCYB_T1 target, 25 plants regenerated on selection-free media were also screened by collecting tissue and pooling the DNA. In all cases, no mutations were detected. While 75 embryos bombarded with sgRNA-gw2 were screened in eight pools. Of these, six pools of the GW2-D amplicon were estimated to be present with indels near the cut site. The ICE v2 Knockout (KO) scores for these pools ranged from 3 to 14. The KO score represents the reads that contain an amino acid frameshift change. The pool containing five embryos had a KO score of 5, which was the second lowest score of all pools predicted to have been edited. For the GW2-B pools, only one pool was estimated to be present with sequences containing indels with a KO score of 3. Interestingly, the sequence contributing to this

knockout score was predicted to have a 2-nucleotide insertion. While the contributing sequences of all the edited pools of GW2-D were estimated as 2-nucleotide deletions, with the majority being deletions immediately after the predicted Cas9 cut site. The GW2-B pool that was present with indels (GW2-B2) had a KO score of 3, while the D-amplicon amplified from the same pool (GW2-D2) contained a KO score of 7. The GW2-A amplicons all returned with KO scores of 0, indicating that no editing took place on the GW2-A1 genome. These results suggest that editing was much more efficient in the GW2-D1 homeolog than in the GW2-B1 copy, and further indicate that no editing occurred on the GW2-A1 copy which contains a single mismatch in the target sequence. While an estimate of editing efficiency cannot be produced in this mutation detection procedure, the projection that the gw2-sgRNA is more efficient at editing *TaGW2-D1* than *-B1* is corroborated by Liang *et al.,* [18]. They used the same target as the present study to produce 28 *gw2* mutant plants, 14 of which contained indels in *TaGW2-B1* and all 28 in *TaGW2-D1*. After preliminary indications that editing had occurred, D-amplicons of the pools predicted by ICE v2 to have been present with indels were subcloned into pGEM-T-easy. Clones were screened by digestion and undigested clones were Sanger sequenced. Twenty GW2-D clones were screened and four did not linearise. The sequences of these clones revealed that they all contained SNPs in the recognition site of the RE *Xba*I. Clone GW2D-3 was present with a G/T substitution immediately following the predicted cut site and GW2-D1 with a A/G substitution 2 bases downstream of the cut site. Two clones (GW2D-8 and -19) possessed a T/C substitution in the PAM site.

Since sgRNAs ultimately must be validated empirically through *in vivo* application, it is recommended that transformed tissue be analysed shortly after the transformation procedure to assess whether the sgRNAs are effective. This can be done because RNPs function almost instantly upon being introduced to the cell and are then swiftly degraded [147]. Furthermore, a rapid screening method could save a substantial amount of resources and time through the early discovery of the effectiveness of a sgRNA target. *In vivo* validation of sgRNAs is often performed through the transfection of protoplasts with RNPs [191–193]. Subsequent analysis through deep-amplicon sequencing is commonly used to detect and characterise indels. This application is limited when trying to implement a breeding program since regeneration of wheat protoplasts is not yet feasible in wheat. It is the opinion of this author that if resources permit, the rapid validation of sgRNAs *in vivo* could better be assessed through RNP biolistics. The early verification of bombarded embryos is often cited in literature [18,186]. However, the procedure is mostly done using high-throughput NGS analysis. Targeted NGS is now widely adopted, and it is the favoured method for indel profiling in genome edited plants. Currently, NGS provides the most comprehensive data on edited sequences. Many individual reads can be obtained for a given target site, providing accurate and sensitive information on indel frequencies and sizes. However, the widespread use of NGS is limited by the labour, time and cost constraints associated with it. Furthermore, numerous factors can affect the efficacy of a transformation event. Considering this, committing to an NGS strategy could be a costly endeavour without a guarantee of positive results. In this study, we decided to employ a

simpler, more cost-effective screening method. Time and effort are saved in the process of pooling embryos prior to DNA extraction. Due to the low editing frequencies reported in past studies using RNPs, it is important to screen a sufficient sample size of each transformation event to detect any mutations. Embryos pooled to avoid having to extract DNA from 100 individual embryos, which could easily become an overwhelming procedure. Once DNA is extracted from batches of embryos, each batch would contain an overwhelming amount of WT sequences relative to mutated sequences. Therefore, PCR amplicons derived from batches are digested using a RE that would enrich for edited sequences. The process of enrichment is important for highly complex genomes where it is possible that only a single allele of the six in total could be edited in an individual. Chimerism of individual embryos could also contribute to the “noise”. Chimeras can be formed by individuals harbouring both mutated and unmutated cells [194]. However, as described, an RE/PCR assay is not sufficient for detecting mutations in many cases. If this is the case, RE/PCR amplicons could be Sanger sequenced and submitted to online deconvoluting software programs such as ICE. This proposed model of detecting indels in CRISPR/Cas9 edited wheat is not intended to be informative about indel frequencies or editing efficiencies. Rather, it provides a simple and cost-efficient validation that could be applied at an early screening stage.

Plant regeneration is essential in any genome-editing application to be implemented in a breeding program. In this study, 25 LCYB_T1 and 23 sgRNA-gw2 plants were regenerated. No editing was detected in the preliminary screening of these plants. It is likely that such small sample sizes are not sufficient to draw any conclusions on the effectiveness of the chosen sgRNAs. However, these results highlight a potential pitfall in using CRISPR RNPs, or transient transformation protocols in general. Without the inclusion of a selectable marker during tissue culture it is to be expected that many plantlets, comprised largely of non-edited plants, will be regenerated. Identifying modified plants would involve screening the DNA of pooled or individual regenerants. This could quickly become unfeasible when screening hundreds of regenerants. The problem is further compounded by the possible formation of chimeras when using selection-free media. Chimerism could be a result of chimeric clusters formed during callus formation, or through chimeric individual plants comprised of both mutated and unmutated cells [194]. Chimeric plants have been produced in previous reports using tissue culture [195,196]. The addition of a selectable marker during transformation facilitates the reduction of chimerism by eliminating untransformed cells [197] and therefore reduces the final number of plants that need to be screened. The results achieved in this study when screening immature embryos suggest a very low editing efficiency, and even the possible presence of chimeras. At such rates, detecting mutant plants would not be feasible through PCR/RE assays or Sanger sequence analysis. Nor would NGS be a viable option without becoming prohibitively expensive.

The aim of this work was to successfully edit immature wheat embryos by using transiently active CRISPR/Cas9 RNP complexes. The results presented indicate that the transformation procedure is a viable approach to edit bread wheat. Further, an accessible method of screening for sgRNA activity

in vivo is described. Despite this, major drawbacks regarding mutant detection and plant regeneration could hinder its application on a larger scale. While the transformation protocol used in this chapter is not practical, alternative protocols for producing non-transgenic wheat with CRISPR/Cas 9 are proposed.

i) Co-bombardment of RNPs with selection-encoding plasmids. This was recently achieved in maize [149] and in rice [148]. In maize, they were able to achieve a transformation efficiency of 47% when RNPs were co-delivered along with a selective marker. Although this is not transgene-free, it could facilitate the identification of mutated plants and transgenes could be crossed out in subsequent generations.

ii) The transient expression of CRISPR/Cas9 DNA (TECCDNA) is a transgene-free transformation method developed by Liang *et al* [17]. It involves the delivery of CRISPR/Cas9 plasmids into immature embryos, but plantlets are regenerated on selection-free media. Through this method the authors were able to produce T0 mutants, with 86.8% of the mutants being transgene-free. This method could address the difficulties in working with *in vitro* synthesised RNA. Therefore, it could improve overall editing efficiency.

Chapter 4: Wheat transformation using a CRISPR/Cas9 DNA construct

4.1 Introduction

The genetic transformation of wheat has historically lagged behind other major cereals. Initial attempts at wheat transformation often took over a year to produce mutant plants at exceedingly low efficiencies of approximately 0.2% [55,198]. The challenge of transforming wheat is largely due to its poor response to tissue culture [198]. Plant tissue culture exploits the cellular totipotency of isolated explant tissue derived from a donor plant to induce callus formation, followed by the regeneration of shoots and roots to ultimately produce fertile plants. Tissue culture is a crucial factor contributing to successful transformation techniques [199]. While some plant species are easily amenable to tissue culture, it has often been challenging to establish and maintain wheat in tissue culture [55,198,200]. Wheat explant tissue used for transformation is often limited to immature embryos which are then induced into embryogenic calli. However, immature embryos are only viable for transformation within a range of a few days. Transformation strategies using alternative explants such as calli developed from the scutella of immature embryos have been developed [201]. Mature embryos have also been used but without great success, however mature embryo culture has been optimised recently and could potentially be utilised more often in the future [199].

The modification of plant genomes has proven to be a powerful tool in functional genomic research and has further facilitated the improvements of crop varieties. Wheat transformation has the potential to improve commercial varieties with beneficial agronomic traits more rapidly than traditional breeding techniques [202]. Numerous transformation methods have been developed for wheat and have been utilised with mixed success. The transformation techniques are categorised as either direct or indirect DNA transformation methods. Direct DNA transfer methods include protoplast electroporation, microinjection, and particle bombardment [203], while indirect DNA transfer is achieved through *Agrobacterium*-mediated transformation [202]. The first reported event of wheat transformation was achieved in 1992 by Vasil *et al.* [*55*] via particle bombardment. *Agrobacterium*-mediated transformation of wheat immature embryos was then accomplished in 1997 by Cheng *et al* [61]. Particle bombardment has been the favoured method of delivering foreign DNA to target wheat tissues because it is simpler to establish compared to using *Agrobacterium*, which requires additional transformation and tissue culture steps [202]. However, particle bombardment does require the use of specialist instruments.

The development of novel genome editing strategies, such as CRISPR/Cas9, offers encouraging opportunities to modify gene expression in wheat. In recent years, genome editing by CRISPR/Cas9 has become the prevailing technique for crop breeding, not merely for agronomic traits, but also in the development of new wheat varieties that could be healthier to consume [13,204]. Furthermore, the CRISPR/Cas9 system has gained increasing utility in crop breeding due to it being easily

adaptable to multiplex strategies that allow for the targeted editing of several genomic loci simultaneously [205–207]. Multiplex editing approaches provides the opportunity to rapidly elucidate gene family functions [208] and could further accelerate the breeding process [209]. For example, Camerlengo *et al*. [208] simultaneously targeted two genes coding for two separate α-amylase/trypsin inhibitors associated with wheat allergies to produce less allergenic durum wheat [210]. In the current study, the establishment of a multiplex genome editing platform in bread wheat was attempted.

4.2 Materials and Methods

4.2.1 Plasmid vector construction

The plasmid pDIRECT_26H was a gift from Daniel Voytas (Addgene plasmid # 91150 ; http://n2t.net/addgene:91150 ; RRID:Addgene_91150) and was used as a backbone for the construction of the Cas9-sgRNA-expressing vector. Briefly, the ZmUbi1 promoter was used to express the Csy4-Cas9 module, and the PvUbi promoter drove expression of the sgRNA processed by Csy4 to release the multiplexed sgRNAs (Figure 4.1a). Golden Gate assembly reactions were performed as described by Čermák *et al* [211]. Primers for the assembly of the sgRNA targets from section 3.3.1 into the pDIRECT_26H vector were designed using the online primer design tool (http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/). The Voytas lab primer design tool accepts multiple sgRNA sequences in fasta format to provide primer sequences containing appropriate overhangs for use in a Golden Gate assembly reaction. Primers for the assembly of the vector are provided in Table 4.1. For the assembly of three sgRNA spacers, four PCR reactions were setup using Phusion DNA polymerase (New England Biolabs, USA) and the target cloning vector as a template (Figure 4.1b). The primer combinations for the reactions were as follows; 1) oPvUbi1 + CSY_target1 2) REP_target1 + CSY_target2 3) REP_target2 + CSY_target3 4) REP_target3 + CSY_term. The PCR cycle conditions were 98°C/1min + 30x (98°C/10sec + 60°C/15sec + 72°C/15sec) + 72°C/2min + 4°C. The resulting PCR products were assimilated into the pDIRECT26_H vector by assembling the following Golden Gate reaction: 50 ng of the pDIRECT_26H vector, 5 ng of each PCR product, 0.5 µL *Sap*I, 0.5 µL *Aar*I (2 U/µL) (Thermo Scientific, USA), 0.4 µL *Aar*I oligonucleotide (0.5 µM) (Thermo Scientific, USA), 1 µL T7 DNA ligase (New England Biolabs, USA), 10 µL 2X T7 DNA ligase buffer, and dH_2O for a total reaction of 20 µL. The PCR cycle was set to: 10x (37˚C/5min + 25˚C/10min) + 4°C hold. The final pDIRECT_26H plasmid, henceforth termed pDIR26H-LCYB, was confirmed by Sanger sequencing at CAF (Stellenbosch University, SA) using primers TC306 and M13F (Table 4.1). The complete plasmid map is provided in Supplementary Figure 3.

Table 4.1 Primers used for cloning and Sanger sequencing of the pDIRECT_26H construct.

Primer Name	Sequence (5'-3')	Amplicon length (bp)
oPvUbi1	TGCTCTTCGCGCCACGTCAGTGTTTGGTTTCC	2040
CSY_target1	TATCACCTGCCCCCCGTCGCTGGTCGCTGCCTATACGGCAGTGAAC	
REP_target1	TATCACCTGCCCCAGACGTCTCCTCGGTTTTAGAGCTAGAAATAGC	148
CSY_target2	TATCACCTGCCCCCATGGTGGCGCGCCTGCCTATACGGCAGTGAAC	
REP_target2	TATCACCTGCCCCACCATGTACCCTGGTTTTAGAGCTAGAAATAGC	148
CSY_target3	TATCACCTGCCCCCGACCGCTGCATCCTGCCTATACGGCAGTGAAC	
REP_target3	TATCACCTGCCCCAGGTCCATCATGGGTTTTAGAGCTAGAAATAGC	134
CSY_term	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC	
TC306	AGCACTACCAATGATGACCT	712
M13F	GTAAAACGACGGCCAGT	

4.2.2 *Agrobacterium*-mediated transformation of immature embryos

4.2.2.1 Agrobacterium-mediated transformation

Agrobacterium-mediated transformation was performed as described by Ishida *et al* [63]. The pDIR26H-LCYB construct was introduced into *A. tumefaciens* strain EHA105 by electroporation. A single positive colony was inoculated into 10 ml of liquid LB media supplemented with 50 µg/ml kanamycin and 50 µg/ml rifampicin and incubated for ~48 hr at 28°C on a shaker at 160 rpm. Glycerol stocks were prepared by mixing equal quantities of the *Agrobacterium* culture and 50% sterile glycerol. The day before wheat transformation, the *Agrobacterium* culture was inoculated in 10 ml of MG/L (all media recipes used in *Agrobacterium*-mediated transformation shown in Table 4.2) [212] without antibiotics and incubated at 28°C on a shaker, shaken at 200 rpm for ~16 hr. The culture was centrifuged at 900 rcf for 10 min at 24°C and resuspended in WLS-inf at an OD (600 nm) to produce the inoculum.

4.2.2.1.1 Explant preparation

Immature embryos were excised as described in section 3.2.5.1. Excised embryos were transferred into 2 mL of WLS-liq medium in a 2 mL microcentrifuge tube at ~ 100 embryos per tube. The tube was inverted several times and the WLS-liq was removed. The 2 mL of fresh WLS-liq was added to the tube and centrifuged at 20 000 rcf at 4°C for 10 min. The WLS-liq medium was removed and the embryos were resuspended in 1 mL inoculum. The tube was then inverted frequently for 30 s and incubated at room temperature (RT) for 5 min. The immature embryos were transferred to WLS-AS plates with the scutellum side up. The plates were incubated at 23°C in the dark for two days.

4.2.2.2 Tissue culture and regeneration

After co-cultivation, embryogenic axes were excised from the embryos using fine forceps and a scalpel. The embryos were transferred to WLS-Res media for 5 days of resting culture. After the resting culture, scutella were transferred to WLS-P5 callus induction media for 2 weeks, followed by WLS-P10 callus induction media for 3 weeks. Calli were then transferred to LSZ-P5 regeneration media for 2 weeks under a cycle of 12 h dark/12 h light (68 µmol/m²/s). Regenerated plants that developed shoots were transferred to LSF-P5 rooting media for 2 weeks.

Table 4.2 Media recipes for media used in Agrobacterium-mediated transformation.

MG/L	5 g/L mannitol, 1 g/L L-glutamic acid, 250 mg/L KH_2PO_4, 100 mg/L NaCl, 100 mg/L $MgSO_4.7H_2O$, 5 g/L tryptone, 2.5 g/L yeast extract, and 1 µg/L biotin; pH=7.0
WLS-inf	0.19 g/L KNO_3, 0.165g/L NH_4NO_3, 44 mg/L $CaCl_2{\cdot}2H_2O$, 37 mg/L $MgSO_4{\cdot}7H_2O$, 17 mg/L KH_2PO_4, 2.23 mg/L $MnSO_4{\cdot}5H_2O$, 1.06 mg/L $ZnSO_4{\cdot}7H_2O$, 0.62 mg/L H_3BO_3, 0.083 mg/L KI, 0.025 mg/L Na_2MoO_4, 0.0025 mg/L $CuSO_4{\cdot}5H_2O$, 0.0025 mg/L $CoCl_2$, 10 mg/L myoinositol, 0.1 mg/L thiamine hydrochloride, 0.2 mg/L glycine, 0.05 mg/L pyridoxine hydrochloride, 0.05 mg/L nicotinic acid, 2.78 mg/L $FeSO_4{\cdot}7H_2O$, 3.73 mg/L EDTA, 10 g/L glucose, 0.5 g/L 2-(N-Morpholino)ethanesulfonic acid (MES), 100 µM acetosyringone (AS), and pH adjusted to 5.8
WLS-liq	WLS-inf without AS
WLS-AS	WLS-inf plus 100 µM AS, 0.85 mg/L $AgNO_3$, 1.25 mg/L $CuSO_4{\cdot}5H_2O$ and 8 g/L agarose
WLS-Res	1.9 g/L KNO_3, 1.65g/L NH_4NO_3, 0.44 g/L $CaCl_2{\cdot}2H_2O$, 0.37g/L $MgSO_4{\cdot}7H_2O$, 0.17g/L KH_2PO_4, 22.3 mg/L $MnSO_4{\cdot}5H_2O$, 10.6 mg/L $ZnSO_4{\cdot}7H_2O$, 6.2 mg/L H_3BO_3, 0.83 mg/L KI, 0.25 mg/L Na_2MoO_4, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2$, 0.1 g/L myoinositol, 1 mg/L thiamine hydrochloride, 2 mg/L glycine, 0.5 mg/L pyridoxine hydrochloride, 0.5 mg/L nicotinic acid, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, 37.3 mg/L EDTA, 0.5 mg/L 2,4-D, 2.2 mg/L picloram, 0.5 g/L glutamine, 0.1 g/L casein, 0.75 g/L $MgCl_2{\cdot}6H_2O$, 40 g/L maltose, 1.95 g/L MES, 0.1 g/L ascorbic acid, 0.25 g/L carbenicillin, 100mg/L cefotaxime, 0.85 mg/L $AgNO_3$, and pH adjusted to 5.8
WLS-P5	WLS-Res without cefotaxime and supplemented with 5 mg/L phosphinothricin (PPT)
WLS-P10	WLS-P5 but supplemented with 10 mg/L PPT
LSZ-P5	1.9 g/L KNO_3, 1.65g/L NH_4NO_3, 0.44 g/L $CaCl_2{\cdot}2H_2O$, 0.37g/L $MgSO_4{\cdot}7H_2O$, 0.17g/L KH_2PO_4, 22.3 mg/L $MnSO_4{\cdot}5H_2O$, 10.6 mg/L $ZnSO_4{\cdot}7H_2O$, 6.2 mg/L H_3BO_3, 0.83 mg/L KI, 0.25 mg/L Na_2MoO_4, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2$, 0.1 g/L myoinositol, 1 mg/L thiamine hydrochloride, 0.5 mg/L pyridoxine hydrochloride, 0.5 mg/L nicotinic acid, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, 37.3 mg/L EDTA, 5 mg/L Zeatin, 20 g/L Sucrose, 0.5 g/L MES, 2.5 mg/L $CuSO_4{\cdot}5H_2O$, 8 g/L agar, pH adjusted to 5.8 and supplemented with 5 mg/L PPT
LSF-P5	1.9 g/L KNO_3, 1.65g/L NH_4NO_3, 0.44 g/L $CaCl_2{\cdot}2H_2O$, 0.37g/L $MgSO_4{\cdot}7H_2O$, 0.17g/L KH_2PO_4, 22.3 mg/L $MnSO_4{\cdot}5H_2O$, 10.6 mg/L $ZnSO_4{\cdot}7H_2O$, 6.2 mg/L H_3BO_3, 0.83 mg/L KI, 0.25 mg/L Na_2MoO_4, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2$, 0.1 g/L

	myoinositol, 1 mg/L thiamine hydrochloride, 0.5 mg/L pyridoxine hydrochloride, 0.5 mg/L nicotinic acid, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L EDTA, 0.2 mg/L IBA, 15 g/L sucrose, 0.5 g/L MES, 5 mg/L PPT, 3 g/L gelrite, and pH adjusted to 5.8

4.2.3 Biolistic bombardment of mature imbibed wheat seeds

4.2.3.1 Explant preparation

Mature seeds of wheat were sterilised by soaking in 6% sodium hypochlorite for 20 min and rinsed numerous times with sterile distilled water. Seeds were placed on moist filter paper at 22°C and allowed to germinate overnight. The parts of the coleoptile and leaf primordia covering the shoot apical meristem (SAM) were excised with a scalpel under a stereomicroscope. Embryos were subsequently separated from endosperms and placed upright on Murashige and Skoog (MS) medium [213] supplemented with 30 g/L maltose, 0.98 g/L MES, 3% plant preservative mixture (PPM) and 7 g/L phytagel, and the pH adjusted to 5.8. Thirty embryos per plate were placed in a circle, approx. 0.8 cm in diameter.

4.2.3.2 Bombardment of apical meristem

To complex the pAHC25 plasmid onto gold microcarriers, a 50 µl aliquot of the gold particles (prepared in section 3.2.5.2) was thawed on ice and then briefly vortexed. Then 5 µg of plasmid DNA was added to the tube and the tube was vortexed for 3 s. 50 µl 2.5 M $CaCl_2 \cdot 2H_2O$ and 20 µl 0.1 M spermidine were added to the mixture and the mixture was vortexed for 5 s. The mixture was then incubated at room temperature for 3 min to allow for the binding of DNA to the gold microcarriers. The mixture was then centrifuged at 16 000 rcf for 5 s and the supernatant discarded. The pellet was then washed in 150 µl 96% EtOH after which it was centrifuged again at 16 000 rcf for 5 s. The supernatant was discarded, and the coated particles were resuspended in 85 µl 100% EtOH and kept on ice prior to shooting. The pAHC25 vector was delivered into the SAMs of mature wheat embryos through particle bombardment as described by Hamada *et al*. [214], using a Bio-Rad PDS-1000 He™ particle gun. The particle gun components were sterilised using 70% ethanol. The macrocarriers and microcarrier holder were sterilised by dipping in 96% EtOH prior to being loaded with 5 µL of DNA-coated gold microcarriers. Once loaded, the macrocarriers were allowed to air-dry for 1 h. The bombardment was done using a 1 350 psi rupture disc with the plate containing the target tissue being placed at a 6 cm target distance. Following bombardment, plates were incubated in the dark at 24°C.

4.2.3.3 Plant growth conditions

Twelve hours after transformation, mature embryos were transferred into a petri dish containing basal MS medium and incubated three weeks under long day conditions (16 h light/8 h darkness) at 22°C. Seedlings were planted in pots and grown in a growth chamber under the same conditions.

4.2.4 Biolistic bombardment of embryogenic calli

4.2.4.1 Explant preparation

Immature embryos were excised as described in section 3.2.5.1 and placed scutellum side up on a petri dish of Callus induction media (CIM) (All media used in the biolistic bombardment of calli shown in Table 4.3). Approximately 80 embryos were deposited per plate. The plates were incubated at 24°C for 7 days in darkness to initiate callus formation. Vigorously growing calli were separated from the scutella using forceps and placed in the central region of fresh CIM plates. 25 calli were put on a single plate (100 x 15 mm) in a 5 x 5 pattern. The plates were once again incubated as before for 2 days before bombardment. On the day of bombardment, the plates were preconditioned by air-drying the calli in a laminar flow hood for 20 min.

4.2.4.2 Bombardment of wheat calli with a CRISPR/Cas9 DNA construct

Complexing of the pDIR26H-LCYB plasmid onto gold particles was done as described in section 4.2.3.2. The vector was delivered into wheat calli following the protocol of Bin Tian *et al* [215] with modifications from Sparks and Doherty [216], using a Bio-Rad PDS-1000 He™ particle gun. The bombardment procedure was as described in section 4.2.3.2 with minor adjustments. For the bombardment of wheat calli a 1 100 psi rupture disc was used.

4.2.4.3 Tissue culture

Tissue culture and media composition was done according to Hayta *et al* [217]. After bombardment, calli were kept on CIM plates. The CIM plates were incubated for 3 days at 24°C in the dark to recover. After 3 days the bombarded calli were transferred to selection 1 media (CIM-s1) and incubated in the dark for 2 weeks at 24 °C. Thereafter, calli were transferred to selection 2 (CIM-s2) containing 10 mg/L PPT and incubated in the dark for 2 weeks at 24 °C. After 2 weeks on CIM-s2 the calli were split into clumps of approximately 4 mm² using forceps and transferred to fresh CIM-s2 plates. The calli were exposed to fluorescent light (100 µmol/m²/s) and covered with a single layer of paper towel. After one week, the paper towel was removed to expose the calli to direct light. After 3 weeks on CIM-s2, wheat calli were transferred to wheat regeneration media (WRM) in deep petri dishes (90 mm diameter x 20 mm) and incubated under fluorescent light (100 µmol/m²/s) at 24°C with a 16-hr photoperiod. Regenerated shoots that were 1 cm in length and had begun to develop roots were transferred to deep petri dishes containing Rooting media (ROM).

Table 4.3 Media recipes for media used in regeneration of calli bombarded with the pDIR26H-LCYB construct.

CIM	MS: 4.4 g/L, Maltose: 30 g/L, Casein hydrolysate: 1 g/L, Picloram: 2 mg/L, 2,4-D: 0.5 mg/L, Thiamine-HCl: 1 mg/L, myo-inositol: 0.35 g/L, proline: 0.69 g/L, $CuSO_4.5H_2O$: 1.25 mg/L, Agarose: 5g/L, pH: 5.8
CIM-s1	same as CIM, but supplemented with 5 mg/L PPT
CIM-s2	same as CIM, but supplemented with 10 mg/L PPT
WRM	MS including vitamins (Duchefa M0221): 4.4 g/L, Sucrose: 20 g/L, MES hydrate: 0.5 g/L, Zeatin: 0.5 mg/L, PPT: 6.5 mg/L, Gelrite: 3 g/L, pH: 5.8
ROM	CIM without the growth regulators picloram and 2,4-D, and supplemented with 5 mg/L PPT

4.2.5 GUS histochemical assay

GUS solution was composed as follows: 3.72 g/L EDTA, 8.8 g/L sodium phosphate monobasic, 0.21 g/L potassium ferrocyanide, 0.1% Triton X-100, 0.5 g/L X-gluc, pH=7.0. Calli were totally submerged in GUS solution in a 2 ml Eppendorf tube and incubated at 37°C overnight. Calli were then observed under a stereo microscope for blue staining.

4.3 Results

4.3.1 Assembly of the CRISPR/Cas9 construct

To construct the genome editing vector, the backbone of pDIRECT26_H was used (Figure 4.1a). pDIRECT26_H contains a *Triticum aestivum* codon-optimised Cas9 gene, driven by the *ZmUbi* promoter, and the *PvUbi1* promoter for the transcription of the multiplexed sgRNA targets. The selection of transgenic plants is facilitated by the *bar* gene, encoding the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to phosphinothricin (PPT), and which is driven by the *PvUbi2* promoter. The *PvUbi1* promoter generates a single transcript containing the three *LCYB* targets chosen in section 3.3.1, each sgRNA is separated by a 20 bp Csy4 recognition hairpin to release individual sgRNAs [211]. PCR fragments containing the spacer sequences were simultaneously assembled into pDIRECT26_H by the Golden Gate assembly method (Figure 4.1b). The assembled T-DNA region of the CRISPR/Cas9 pDIR26H-LCYB vector with Csy4-processing segments are shown in Figure 4.1c. The sequencing results confirming successful assembly is in Supplementary Figure 4.

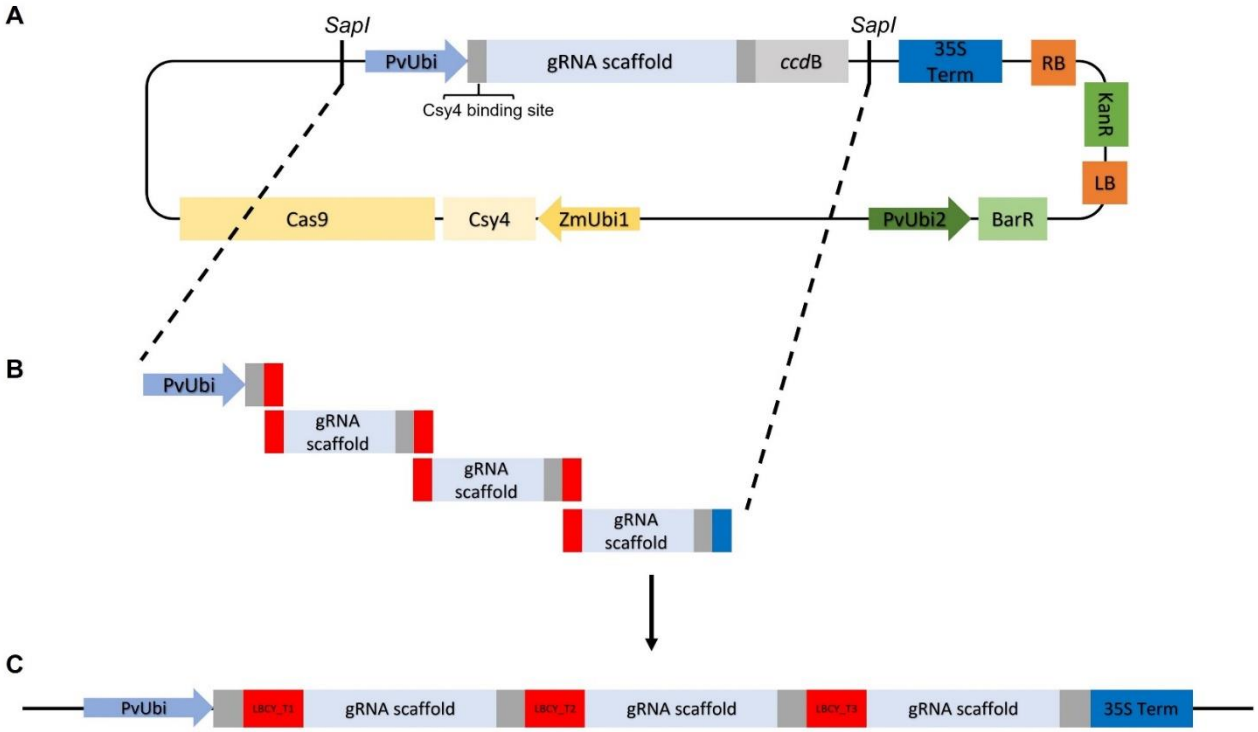


Figure 4.1. A schematic diagram of the CRISPR/Cas9 pDIRECT26_H system with the multiple sgRNA expression cassette. A) The structure of the direct cloning vector pDIRECT26_H is designed to enable the rapid assimilation of sgRNAs on a single T-DNA cassette also expressing the Cas9 endonuclease. BarR: phosphinothricin acetyltransferase (PAT), PvUbi: Panicum virgatum ubiquitin 1 promoter, ccdB: lethal gene in E. coli, PvUbi2: Panicum virgatum ubiquitin 2 promoter, ZmUbi1: Zea mays ubiquitin promoter, Csy4: RNA endoribonuclease Csy4 from Pseudomonas aeruginosa, Cas9: Cas9 endonuclease, Csy4-binding site illustrated in grey represents a 20 bp sequence recognised by Csy4. SapI RE cut sites are exploited to insert the multiple sgRNA expression cassette. B) Illustration of cloning of multiplexed sgRNA cassettes into the CRISPR/Cas9 pDIRECT_26H vector by the Golden Gate assembly method. The 20nt protospacer sequences are overlapping regions for assembly, shown as red boxes. C) Final configuration of the multiplexed sgRNAs targeting the LCYB gene in the pDIR26H-LCYB vector.

4.3.2 *Agrobacterium*-mediated transformation of immature embryos

The pDIR26H-LCYB construct was delivered into immature embryos of wheat cv. Bobwhite via *Agrobacterium*-mediated transformation as described by Ishida *et al* [63]. As a control, the plasmid pBRACT204 [218] was used in concurrent experiments. The pBRACT204 plasmid contains the *uidA* gene, encoding the ß-glucuronidase (GUS) enzyme (plasmid map and details can be found at www.bract.org). Two transformation events (Agro 1 and Agro 2) were conducted as summarised in Table 4.4. A total of 109 embryos were transformed with the pDIR26H-LCYB plasmid, from which a total of three regenerants were obtained. None of the three regenerants were found to be positive for the *Ta*Cas9 insert when screened with PCR, using primers TaCas9-F and -R (sequences in Supplementary Table 1). Furthermore, sequence analysis of the LCYB fragment containing the targets on the ICE v2 software returned no edited sequences [158] (ICE v2 results provided in Supplementary Figure 5).

Table 4.4 Summary of the Agrobacterium-mediated transformation events conducted on immature embryos.

Transformation event	Construct used for transformation	Total number of embryos	Total number of regenerants	Transgenics (PCR positive)	Transient GUS expression efficiency*
Agro 1	pDIR26H-LCBY	67	2	0	NA
	pBRACT204	31	1	0	6/22 (27.2%)
Agro 2	pDIR26H-LCBY	42	1	0	NA
	pBRACT204	15	NA	NA	10/14 (71.4%)

*GUS assays performed on embryos 2- and 7-days post-transformation for experiments Agro 1 and Agro 2, respectively. Embryos containing blue stains were classed as transiently expressing GUS.

In the control, 46 embryos were transformed in total with the pBRACT204 plasmid. In the first event, GUS assays were performed two days after transformation with 27.2% of screened embryos being positively stained. In the second event, the staining was done seven days after transformation and a staining of 71.4% was observed (Figure 4.2). One regenerant was produced in the Agro 1 event, which was PCR-screened for the GUS insert using the primer pair GUSpB204-F and -R (sequences in Supplementary Table 1) and found to be negative. In the event Agro 2, all embryos transformed with the pBRACT204 plasmid were used in a GUS assay and were therefore not available for regeneration.

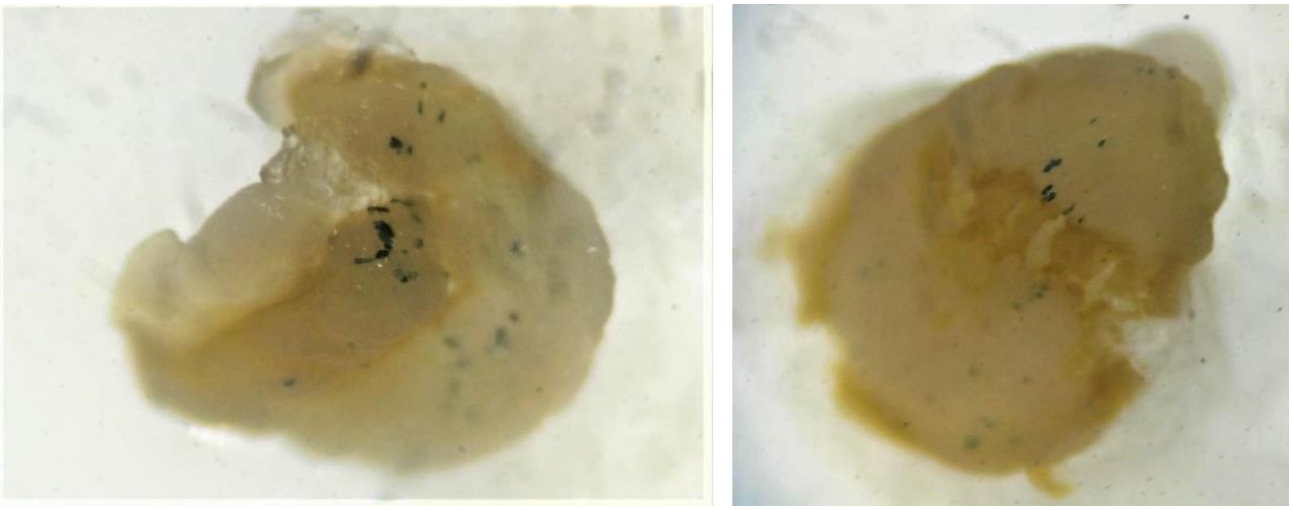


Figure 4.2. Transient expression of GUS in immature embryos transformed with the vector pBRACT204.

4.3.3 Biolistic bombardment of mature wheat seeds

The technique described by Hamada *et al*., was trialled in this study using the GUS-expressing pAHC25 plasmid. Sixty imbibed seeds with their SAMs exposed were bombarded and subsequently 25 were subjected to a GUS assay. Of the 25 seeds, 12 (48%) were positive for GUS expression (Figure 4.3). The remaining embryos were left on basal MS media for 3 weeks to grow. Of the remaining 35 embryos, 12 plants were produced.

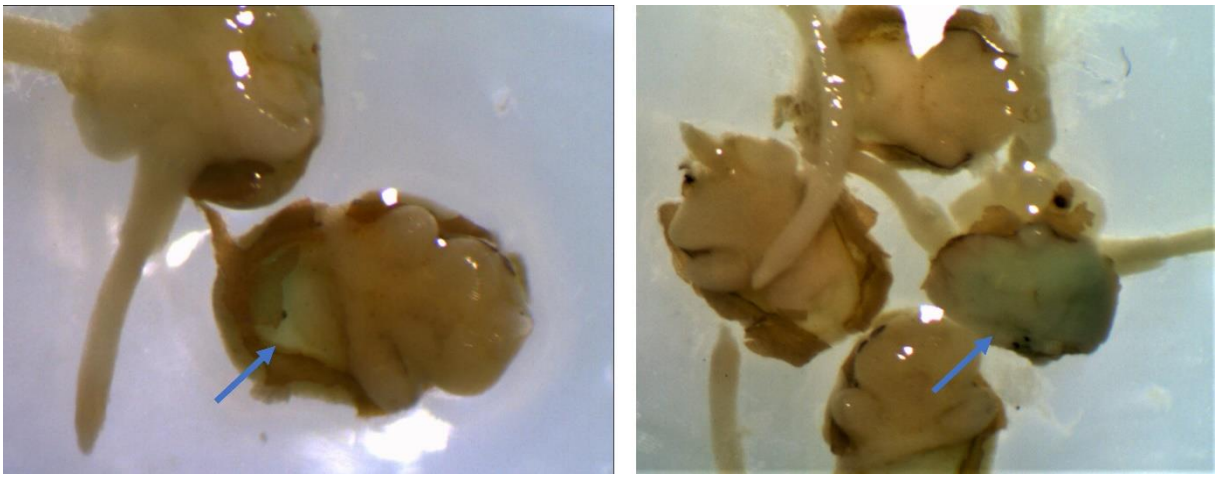


Figure 4.3. Transient expression of GUS in mature wheat seeds. GUS assay was performed two days after bombardment. Blue arrows indicate mature seeds expressing GUS.

4.3.4 Biolistic bombardment of calli with a CRISPR/Cas9 DNA construct

The plasmid pDIR26H-LCYB that was designed for the *Agrobacterium*-mediated transformation was utilised for the particle bombardment of wheat calli. Calli are induced prior to being bombarded with the construct. Callus induction and regeneration was performed as described by Hayta *et al* [217]. Results of the procedure for constructs pDIR26H-LCYB and pAHC25 are summarised in Table 4.5.

Table 4.5 Summary of the results obtained from the biolistic bombardment of induced calli using DNA constructs.

				GUS assay efficiency				
Event name	Construct introduced	Total number of embryos	Total number of screened calli	10 days	30 days	PCR confirmation	Calli containing indels	Regenerants
Calli bomb	pAHC25	100	52	10/13	9/39	4	NA	0
	pDIR26H-LCBY	100	12	NA	NA	NA	2	0

The plasmid pAHC25 [219] was used to optimise the bombardment process. The construct pAHC25 contains the *bar* gene as selectable marker, which encodes for the enzyme phosphinothricin actetyltransferase and confers resistance to the herbicide PPT, and the GUS reporter gene (*uidA*)

encoding the ß-glucuronidase enzyme. Bombarded calli were randomly subjected to a GUS assay at two time points. At 10 days after the event, whole calli were stained at an efficiency of 76.9% (Figure 4.4a). Thirty days after bombardment, 39 calli pieces were subjected to a GUS assay and 9 (23%) were deemed to stain blue (Figure 4.4b).

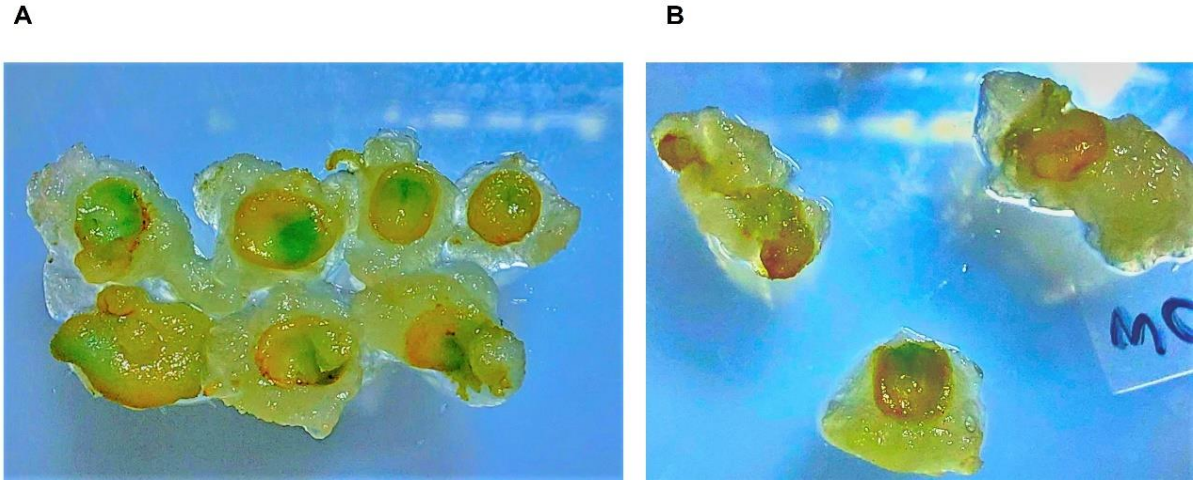


Figure 4.4. GUS-stained calli bombarded with the plasmid pAHC25. A) 10 days post bombardment B) 30 days post bombardment.

DNA was extracted from those that had stained blue and subjected to PCR using primers specific for the *bar* gene (Supplementary Table 1). Four of the calli were present with feint to moderate bands at the expected size of 444 bp (Figure 4.5). The band corresponding to the correct size of calli 4 was sequenced and it was confirmed to be the *bar* gene (Supplementary Figure 6).

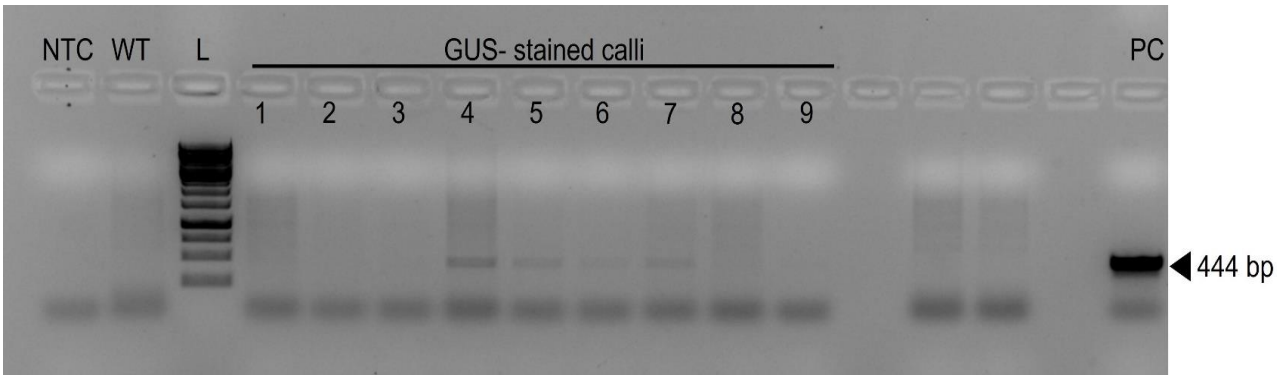


Figure 4.5. PCR screening of GUS-stained calli using bar-specific primers (444 bp). 1% (w/v) agarose gel of the nine calli that stained blue 30 days after transformation. WT: wild-type T. aestivum DNA sample; NTC: No template control; PC: pAHC25 plasmid control; L: 1kb molecular weight marker (GeneRuler, Thermo Scientific).

DNA was extracted from 12 individual calli bombarded by the pDIR26H-LCYB plasmid three months after transformation. LCYB amplicons were generated using primers LCYBint-F and -R (Figure 4.6) (primer sequences in Supplementary Table 1) and visualised on a gel. Amplicons that appeared to have aberrant amplification profiles were excised and digested with *Psp*XI, which has a recognition site spanning the predicted cut site of the LCYB-T1 target. Undigested bands were Sanger

sequenced and analysed using the online software ICE v2. The relevant results are illustrated in Table 4.6.

Table 4.6 ICE v2 software inferences of selected sequences of LCYB amplicons derived from calli bombarded with the pDIR26-LCYB construct.

Sample	Total indel %	Knockout score	Indel Range (bp)
26H-2	10	7	-12 to +12
26H-3	0	0	0
26H-5	35	23	-27 to +9

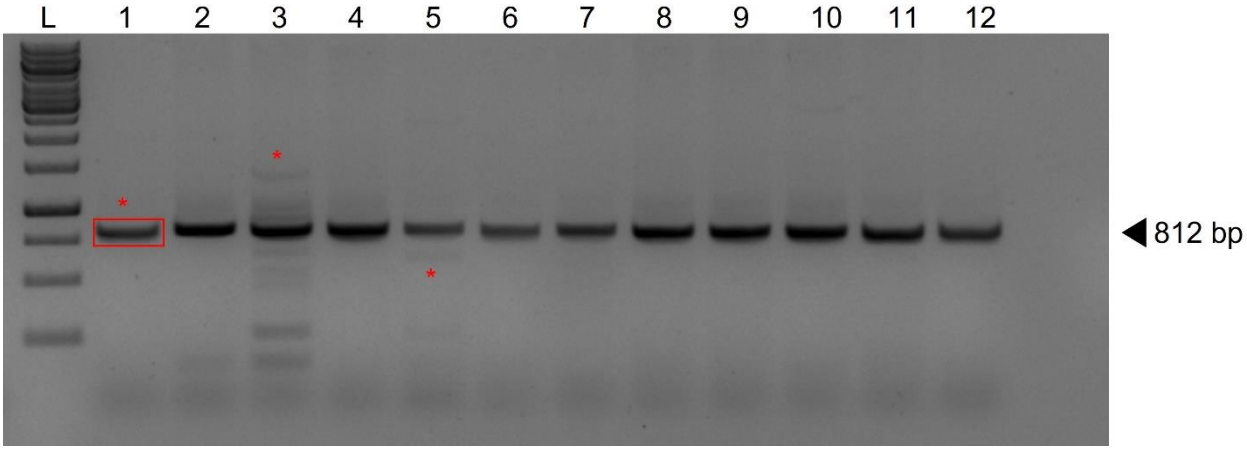
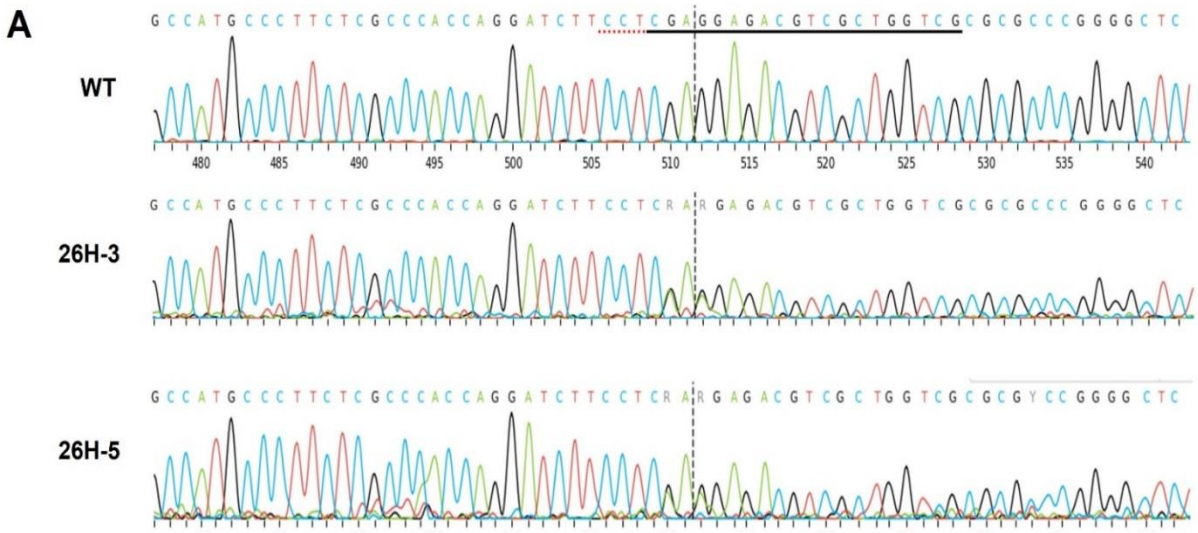


Figure 4.6. LCYB amplicons (~812 bp) derived from calli bombarded with the pDIR26H-LCYB vector. Lanes of interest are denoted with a red asterisk. 1: Slight size difference compared to the rest of the amplicons. 3: Aberrant amplification. 5: Aberrant amplification. Relevant sequence information on the band in the red rectangle (lane 1) is shown in Figure 4.8.

Two amplicons, derived from 26H-2 and 26H-5, showed altered sequences at the LCYB-T1 target site when analysed by ICE v2 (Figure 4.7a). For the 26H-2 callus, the WT sequence contributed 88% of all sequence reads (Figure 4.7b). The sequences had an indel range of -12 to +12 bp, with the most common edited sequence containing a 4 bp deletion spanning the predicted LCYB-T1 cut site. The total indel percentage for 26H-2 was 10%, with a Knockout (KO) score of 7. For the callus termed 26H-5, 59% percent of contributing sequences were determined to be the unedited WT sequence while the remaining contributing sequences were present with indels ranging from -27 to +9 bp. A +1 bp indel immediately following the cut site was the most common type of edit at 12% (Figure 4.7b). The total indel percentage was 35 and ICE v2 estimated a KO score of 23. Both calli were predicted to be present with only WT sequences at the LCYB-T2 target site.



B

26H-2

Indel	Contribution	Sequence
0	88%	TCTCGCCCACCAGGATCTTCCTCGA|GGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-4	3%	TCTCGCCCACCAGGATCTTCCTC--|--AGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-2	2%	TCTCGCCCACCAGGATCTTCCTCGA|--AGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-12	2%	TCTCGCCCACCAGGATCTTCCTC--|----------CTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
+7	1%	TCTCGCCCACCAGGATCTTCCTCGA|nnnnnnnGGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
+10	1%	TCTCGCCCACCAGGATCTTCCTCGA|nnnnnnnnnnGGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
+12	1%	TCTCGCCCACCAGGATCTTCCTCGA|nnnnnnnnnnnnGGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG

26H-5

Indel	Contribution	Sequence
0	59%	TCTCGCCCACCAGGATCTTCCTCGA|GGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
+1	12%	TCTCGCCCACCAGGATCTTCCTCGA|nGGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
+9	7%	TCTCGCCCACCAGGATCTTCCTCGA|nnnnnnnnnGGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-13	3%	TCTCGCCCACCAGGATCTTCCTC--|----------TGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-22	3%	TCTCGCCCACCAGGATCT------|----------CGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-10	2%	TCTCGCCCACCAGGATCTTCCTCGA|----------CTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-12	2%	TCTCGCCCACCAGGATCTTCCTC--|----------CTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-10	1%	TCTCGCCCACCAGGATCTTCCTC--|--------CGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-12	1%	TCTCGCCCACCAGGATCTTCCT----|--------GCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-6	1%	TCTCGCCCACCAGGATCTTCC------|--AGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-19	1%	TCTCGCCCACCAGGATCTTCC------|--------------CGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
-27	1%	TCTCGCCCACC----------|----------GTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG
+6	1%	TCTCGCCCACCAGGATCTTCCTCGA|nnnnnnGGAGACGTCGCTGGTCGCGCGCCCGGGGCTCTCCATGGACGACATCCAGGAGCGCATGGCCGCGCGSCTGAGG

Figure 4.7. ICE v2 software outputs on LCYB sequences of two calli predicted to be present with mutated sequences at the LCYB-T1 target region. A) Trace chromatograms of the region surrounding the LCYB-T1 target for the two calli for which indels were detected by ICE v2. The PAM is underlined by a dotted red line. LCYB-T1 target sequence is underlined in black. Vertical dotted line indicates the predicted cut site. B) Sequence read breakdown inferred by ICE v2. PAM underlined in black. LCYB-T1 target sequence is in red. Short, red vertical lines indicate the predicted cut site. Indels displayed in grey "n" or "-".

The Sanger sequence derived from the amplicon of callus 26H-1 (Lane 1 in Figure 4.6) was not of adequate quality for analysis by ICE v2. However, it was aligned to the WT sequence and presented with mismatches in the region spanned by the LCYB-T2 target (Figure 4.8).

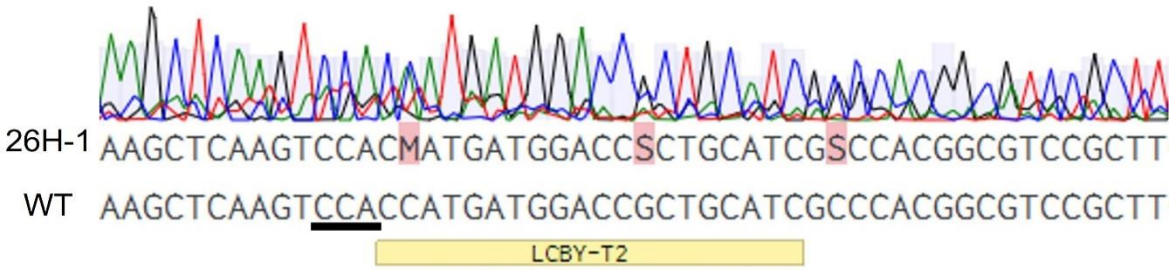


Figure 4.8. Close up on the region surrounding the LCYB-T2 region of the callus 26H-1. PAM sequence is underlined. Red squares indicate mismatches with the wild type.

No regenerants were produced for either the pDIR26H-LCYB or pAHC25 bombarded explants.

4.4 Discussion

This study explored a range of transformation and regeneration techniques in an attempt to produce CRISPR/Cas9 edited wheat plants. Although no mutated plants were regenerated, the results of the experiments conducted, demonstrated the successful editing of wheat and provide insights on general transformation procedures.

Three sgRNAs targeting the *LCYB* gene were assimilated into a single plasmid construct. CRISPR/Cas9 is especially suitable for a multiplexed gene editing approach as target specificity is directed by short sgRNA sequences rather than proteins that have to be engineered for each separate target [205]. The simultaneous expression of multiple sgRNAs is most commonly driven by promoters that regulate the expression of each individual sgRNA [220–222]. However, since it is preferable to use a single vector in plant transformation applications, the addition of several promoter sequences to regulate the expression of each sgRNA individually would exceed the cargo capacity limit of the expression vector and possibly inhibit its downstream application. Recently, several systems have been developed that allow for the expression of multiple sgRNAs from a single transcript. These polycistronic mRNAs are post-transcriptionally processed into individual mature sgRNAs by RNA-cleaving enzymes. These enzymes include tRNA processing enzymes [206], ribozymes [223], and *Csy4* which is a CRISPR-associated RNA endoribonuclease from *Pseudomonas aeruginosa* [224]. The pDIRECT_26H vector was constructed by fusing multiple sgRNAs into a single transcript with each sgRNA being provided with a *Csy4* excision site at their 3' end. The *Csy4* enzyme was co-expressed on the same plasmid to produce three individual sgRNAs that could target multiple sites. This method allows for the simple and rapid assembly of multiple targets that can be co-expressed with the *Csy4* endoribonuclease and the Cas9 endonuclease on the same vector. The establishment of a multiplex genome editing (MGE) approach for bread wheat in this study could serve many beneficial future applications. Although a single sgRNA is often adequate to accomplish editing, targeting multiple sites on a single gene increases the likelihood of

successful editing and the consequent knockout of the gene. Furthermore, MGE designs could also greatly facilitate a more rapid validation of sgRNA efficiencies. Since sgRNAs are still best validated empirically [187], several sgRNAs targeting different sites on the same gene could be assembled onto a single construct and introduced to cells. The relative editing efficiencies of the sgRNAs can then be determined and the best performing sgRNAs selected. Furthermore, whole genes or large parts of genes can be deleted from the genome using MGE. Large deletions of 245 bps using multiple targets have been reported in rice [89], while a 450 bp deletion was achieved in the *Arabidopsis* gene *AGAMOUS* [225]. Eliciting larger deletions could be a more dependable means of achieving a gene knockout. Additionally, mutant plants containing large deletions of a hundred base pairs or more could be more readily screened in PCR assays since the size difference compared to the WT would be easier to visualise through gel electrophoresis. MGE can also be adapted to target multiple genes. This has been done in recent studies to elucidate gene functions in biosynthetic pathways [204] and has the potential to expedite breeding programs by inducing mutations at several genes in one generation.

Agrobacterium and particle bombardment are typically used for the delivery of CRISPR/cas9 reagents to wheat immature embryos [226]. After the delivery of GE components, additional steps are required to regenerate whole, modified plants from the somatic tissue [27]. Plant regeneration underpins most transformation approaches; after transgenes are delivered to isolated immature embryos, selection for the transgene and the regeneration of modified tissues into transgenic plants follow. Several tissue culture protocols focusing on regeneration efficiency have been published for wheat [63,227]. These protocols are largely concerned with optimising media compositions and conditions for *Agrobacterium* infections or bombardment parameters. However, tissue culture protocols are often not effectively transferable between labs and as a result the reported transformation efficiencies are not regularly reproducible [227]. In this study, *Agrobacterium*-mediated transformation and the subsequent tissue culture steps were performed according to Ishida *et al* [63]. For the pBRACT204 control plasmid, in the experiment Agro 1, 22 embryos were subjected to a GUS assay two days after the transformation event. Of the 22 embryos, 6 embryos contained blue staining representing a transient expression efficiency of 27.2%. In the Agro 2 event, a GUS assay was performed on 14 embryos seven days after the transformation event. Of these, 10 embryos were present with blue staining for a transient expression efficiency of 71.4%. The transient expression efficiency of the Agro 2 experiment was dramatically higher than that of Agro 1. As is reported in literature, the size of immature embryos at the time of transformation has been reported as an important factor influencing transformation efficiency [58]. The difference in efficiency observed between the two events can be attributed to insights that were provided by the Agro 1 experiment, in which embryos of variable sizes were subjected to transformation resulting in a low observed transient expression efficiency. These insights informed the Agro 2 procedure and only embryos that were 1-2 mm in diameter were used in this event, which resulted in a much higher observed transient expression efficiency.

Immature embryos transformed with the pDIR26H-LCYB plasmid were left on media to regenerate. In total, 109 immature embryos were subjected to *Agrobacterium*-mediated transformation with the pDIR26H-LCYB plasmid. A total of three regenerants survived the entire tissue culture procedure. The three regenerants, however, did not contain the Cas9 insert, as was established through PCR. The ICE analysis also predicted no indels present in the sgRNA target regions. The lack of regenerated plants reflects the failure of the introduced T-DNA to integrate into the target tissue's genome, as the T-DNA segment would also elicit a resistance to the antibiotics used for selection. Nevertheless, it also highlights the two main bottlenecks limiting the production of transgenic wheat plants; genetic transformation and plant regeneration [27]. Immature embryos are the preferred explant material for genetic transformation in most cereals, including wheat, as the scutella tissue is considered to be competent for both transformation and regeneration [228,229]. The lack of transformation events experienced in this study is likely explained by the differences in explant conditions. As specified in the original protocol by Ishida *et al*., one of the essential factors for achieving highly efficient wheat transformation is the quality of immature embryos [63]. The production of healthy immature embryos is contingent on the use of consistent growing conditions, which could be challenging to regulate year-round.

To address transformation efficiency concerns that can be attributed to the immature embryos' poor response to callus induction, immature embryos were placed on induction media and once calli were formed, healthy calli were bombarded with the pDIR26H-LCYB vector. A biolistic approach was considered suitable as it is more amenable for diverse tissue types [230]. After callus induction and bombardment, the calli were allowed to regenerate on media including selection. The utilisation of embryogenic calli as explant material could increase overall transformation efficiency since calli that are not responsive to tissue culture can be removed from further experimentation and bombardment is performed only on calli that have already responded to regenerative media. Calli that were bombarded with the pAHC25 plasmid were screened on two separate occasions, with the latter screening occurring 30 days after transformation. The positive PCR screening for the *bar* insert obtained from some GUS-stained calli and the confirmation by Sanger sequencing, supported the assumption of the successful stable integration of the GUS and *bar* gene in some of the bombarded calli, lending confidence to a similar result in the pDIR26-LCYB experiment. However, after a prolonged period on selective media, plants failed to regenerate for both constructs, pAHC25 and pDIR26H-LCYB. Calli bombarded by the pDIR26H-LCYB plasmid had largely turned necrotic. Nonetheless, DNA was extracted from twelve that appeared relatively healthy. LCYB amplicons were subjected to digestion with *Psp*XI and further analysed using ICE v2. Two calli, 26H-2 and 26H-5, showed a range of altered sequences at the LCYB-T1 target site. Interestingly, ICE v2 inferred that no edits had taken place at the target site of LCYB-T2. However, this does not conclude that no editing happened at this site, rather it should be noted that the sequences subjected to analysis were those that were treated with the RE *Psp*XI, therefore the remaining sequences would be enriched for sequences edited at the LCYB-T1 site. Further of interest is the presence of a band that is shorter

than the expected amplicon size (lane 1 in Figure 4.6). Although a conclusive sequence was not attained from this sample, its presence does highlight the potential of a multiplexed CRISPR/Cas9 strategy to facilitate the mutant screening process.

The introduction of CRISPR/Cas9 components into wheat tissues is still primarily done through the transformation methods of *Agrobacterium*-mediated or biolistics of DNA constructs – both leading to stable integration of the genes encoding these components [16]. The stable transformation of plants is contingent on two prerequisites, the entry of the construct into cells and the subsequent integration into the host genome. In this study, no evidence was found for the stable transformation of CRISPR/Cas9 components in any of the procedures attempted. This can be concluded with some confidence due to the lack of regenerating plants, as well as the negative PCR screening for the pDIR26H-LCYB plasmid DNA. However, the results of the screened calli do suggest that the pDIR26H-LCYB vector likely acted transiently to introduce mutations in the target tissue. This is possible because once the plasmid enters the cell, it can elute from the microcarrier gold particles and either integrate into the host genome to be expressed stably, or remain as extrachromosomal material and be expressed transiently [231]. This latter facet of exogenous DNA delivery has recently been exploited by Zhang *et al*., [17] in a technique termed the transient expression of CRISPR/Cas9 DNA (TECCDNA). They delivered vectors encoding for CRISPR/Cas9 components into wheat embryos through particle bombardment and proceeded to regenerate plantlets on selection-free media. The authors were able to produce 30 *tagw2* mutants from a total of 640 bombarded embryos for a transformation efficiency of 4.7%. In the current study, it is possible that the CRISPR/Cas9 components on the pDIR26H-LCYB plasmid were transiently expressed and did not integrate into the host genome. Furthermore, the ICE v2 software analysis also suggests that individual calli that were edited, possess a high degree of chimerism at the sgRNA1 target site with 12 different sequences predicted to contribute to the overall read sequences. Chimerism is likely since transformation took place after cell division was initiated. Furthermore, the transient expression of CRISPR elements can lead to chimerism as has been shown previously [172,232]. This can be offset using selective markers which should allow only transformed cells to regenerate, however this would require the stable integration of the selective markers, which was not achieved in this study.

Two protocols for the regeneration of wheat plants from immature wheat embryos were attempted in the experiments discussed above. Although it is possible to improve transformation efficiency by selecting for responsive calli, as was done in the experiment in which we bombarded the DNA constructs, it does not avoid further tissue culture procedures and subsequent selection steps. Tissue culture can be a tedious and time-consuming process, often taking months to complete. Furthermore, it usually requires the addition of expensive selective reagents to the growth medium to regenerate transgenic plants [80]. To bypass the bottleneck experienced in tissue culture-based transformation methods, researchers have attempted to develop *in planta* transformation techniques [233,234]. More recently, Hamada *et al.,* [214] presented a protocol where plasmids expressing Cas9

and an sgRNA were biolistically introduced into the shoot apical meristem (SAM) of imbibed mature wheat seeds. They achieved a transformation efficiency of 5.2% in the T0 generation and 1.4% in the T1 generation. Crucially, since this approach is performed on mature seeds, it does not require callus induction, regeneration, or antibiotic selection. The mechanism of transformation in this technique is based on the introduction of the CRISPR/Cas9 components in the subepidermal (L2) cells that are found in the SAM of mature wheat seeds. Studies have found that the L2 cells differentiate into germ cells such as pollen and egg cells [235,236]. Genome modifications that occur in the L2 cells of the SAM can therefore be passed onto the next generation through germ cell development. This method of plasmid delivery was preliminarily trialled in the current study using the plasmid pAHC25, achieving an estimated 48% transient transformation efficiency for GUS expression. Furthermore, 12 plants were regenerated while further molecular analysis on the regenerants and their progenies was not possible due to restrictions imposed by the COVID-19 pandemic. However, the preliminary results could be interpreted with optimism. Achieving 12 screenable plants from a small starting sample represents an improvement against the tissue culture-based applications attempted in this study. This technique, with further optimisation, could be implemented with a larger number of starting samples to yield transformed plants. It should be noted that although this method has the potential to increase overall transformation efficiency, it does require challenging explant material preparation steps such as the excision of the coleoptile and leaf primordia to expose the SAM.

Chapter 5: Conclusion

5.1 Summary

Since its establishment as an efficient means to induce targeted mutations, considerable effort has been directed at harnessing the potential of the CRISPR/Cas9 system in bread wheat. CRISPR/Cas9 approaches are well suited to genome editing in wheat because of the system's relative simplicity, efficiency, and multiplexing potential. However, bread wheat remains a challenging crop to edit because of its poor response to tissue culture procedures and its recalcitrance to transformation. Therefore, the purpose of this study was to establish an efficient method of CRISPR/Cas9-mediated genome editing in bread wheat.

Chapter 3 of this thesis tested the feasibility of using CRISPR/Cas9 RNPs to produce non-transgenic edited wheat plants following a protocol described by Liang *et al* [18]. The authors presented an appealing approach to developing transgene-free mutants in the first generation that could avoid the use of selective agents in tissue culture and further circumvent the need to cross mutants to eliminate exogenous DNA. However, eliciting targeted mutations through RNPs is not yet a widespread application of CRISPR/Cas9 technology, as most research using CRISPR/Cas9 in wheat is still conducted through the stable transformation of immature embryos. Extensive validation of the RNP technique is still required. In the current study, RNPs targeting the *TaLCYB* gene were delivered into immature embryos of wheat. The early screening of bombarded embryos indicated that no editing had occurred in this gene. However, it was found that immature embryos bombarded with RNPs targeting a previously validated control gene, *TaGW2*, were likely edited albeit at a very low predicted efficiency. As a result, no mutant plants were regenerated. The screening of possible mutations is most comprehensively done through NGS. In the current study, an optimised screening procedure involving pools of embryos being analysed through the online software ICE v2 was employed. This strategy allowed for the rapid detection of putative edits that did not require the costly and time-consuming application of NGS.

The second research chapter (Chapter 4) of this thesis endeavoured to produce wheat plants stably transformed with a DNA construct carrying CRISPR/Cas9 components targeting the *LCYB* gene. The stable transformation of wheat is still the preferred method of obtaining mutant plants. However, this requires tedious experimental practices and time-consuming regeneration protocols. No stable transformants were produced. Editing of the *LCYB* gene was detected in calli that were bombarded with the CRISPR/Cas9 DNA construct. The edited wheat calli were likely acted on transiently, which would partly explain the lack of regeneration on selective media. Furthermore, the introduced DNA construct included three spacer sequences targeting the same gene. Following a multiplex approach, it is possible to increase the efficiency of CRISPR/Cas9 applications. A multiplex system targeting a single gene could also facilitate the screening of mutants by producing sequences of different length compared to the wild type.

The findings of chapter 3 and 4 together highlight the necessity of developing and further optimising transformation procedures, including critical processes such as tissue culture and screening. Numerous strategies were deployed regarding transformation approaches and regeneration protocols. The fact that not a single mutant wheat plant was regenerated illustrates that current protocols are not yet sufficiently reproducible and needs further optimisation to produce wheat mutants at an efficient rate.

5.2 Future considerations

The success of CRISPR/Cas9 experiments is largely determined by the chosen sgRNA sequence. The efficiency of a given sgRNA at guiding Cas9-mediated editing is largely affected by the secondary structure of the guide sequence and the chromatin accessibility of the target sequence [237]. The selection of target sites in the current study was partly facilitated using online software tools designed to determine the most efficient target sites. Numerous sgRNA selection tools have been developed for use in plants. However, a recent study evaluating sgRNA efficiencies determined by different online tools found no significant correlation between the output rankings and the *in vivo* editing efficiencies in *N. benthamiana* [238]. Similarly, a comparison of four different guide design tools found no predictive efficiency measurement when testing six sgRNAs in wheat [239]. Notably, the algorithms that inform most online sgRNA selection tools are developed based on results from animal models [238]. A web-based sgRNA design tool called CRISPR-Cereal [240] (available at http://crispr.hzau.edu.cn/CRISPR-Cereal/) has recently been developed. This software incorporates gene expression profile and chromatin status information to determine sgRNA efficiencies for use in rice, maize, and wheat. Utilising the CRISPR-Cereal tool could greatly improve target selection and consequently editing efficiency using CRISPR/Cas9 applications in wheat.

Bread wheat is a polyploid plant species containing a very large, complex genome consisting of A, B, and D sub-genomes. As a result, the creation of mutations at multiple genomic loci simultaneously is challenging. As was discussed in the current study, the establishment of an efficient multiplex genome editing platform could be a valuable asset for wheat genetic improvement and functional genomic studies. Considering the promising results achieved in this study while employing a multiplex strategy, further optimisation through a comparison of vector components, including promoters driving the expression of Cas9 proteins and sgRNA cassettes, as well as sgRNA processing elements could contribute to the establishment of more efficient multiplex strategies in wheat. For example, Luo *et al.,* [209] recently employed a polycistronic tRNA approach to process multiple sgRNAs simultaneously. A single transcript unit comprised of up to five sgRNAs designed to target five different genes and regulated by a rice *Actin* promoter was introduced into wheat immature embryos through particle bombardment. The researchers were able to recover transgene-free plants that were edited at each gene in the first generation. The scope of multiplex genome editing in plants could further be expanded through the employment of various Cas nickases with various PAM sequence demands such as xCas9, SaCas9, SpCas9-NG, SpG, SpRY, and Cas12a

[241,242]. Enhancing the flexibility and applicability of multiplex strategies would considerably facilitate research in wheat, allowing researchers to detangle complex traits affected by multiple genes more rapidly.

The efficient delivery of CRISPR/Cas9 reagents is a prerequisite for successful genome editing. However, the typical systems of plasmid DNA delivery, such as *Agrobacterium*-mediated delivery particle bombardment, are often limited to a narrow range of regeneratable plant genotypes [243]. The regeneration of transformants or genome-edited explants could theoretically be improved through the expression of plant genes implicated in developmental processes [27]. These genes include developmental regulators referred to as boosters that redirect somatic cells towards embryogenic cell development and drive the regeneration of transformed explants [244]. Overexpression of the boosters *BABY BOOM (Bbm)* and *WUSCHEL2 (Wus2)* has been shown to improve the regenerative ability in a number of transformation-recalcitrant maize genotypes [245–247]. Plant regeneration is therefore expected to be greatly facilitated by the expression of the appropriate booster genes. In a preliminary report, researchers found that the overexpression of a regeneration-related gene *TaCB1* dramatically improved the regeneration efficiency of 31 hexaploid wheat cultivars [248]. They further stated *TaCB1* overexpression could enhance the transformation efficiency of model wheat varieties such as Fielder to up to 90%. Plant-specific transcription factors such as the GROWTH-REGULATING FACTOR (GRF) proteins are involved in cell proliferation and size [249–253]. GRF proteins interact with cofactors, known as GRF-INTERACTING FACTORs (GIFs), to form a GRF-GIF transcriptional complex *in vivo* [254]. GRF-GIF complexes affect proliferation and cell formation during organogenesis [249,254]. A recent breakthrough development for wheat transformation combined the wheat transcription factor GRF4 with its cofactor GIF1 as a protein fusion [255]. Overexpression of the protein fusion substantially improved regeneration of wheat cells growing *in vitro*. Furthermore, the researchers were able to achieve a transformation efficiency of 77.5% when the protein fusion was overexpressed in the wheat variety Fielder. Xue *et al*., [256] were able to expand on these findings by transiently expressing the TaGRF4-TaGIF1 complex in wheat immature embryos. A plasmid encoding the complex was co-bombarded with a cytosine base editor, targeting *TaALS* resulting in a 2-9-fold increase in the regeneration of 11 transgene-free edited wheat cultivars. As illustrated by these recent studies, the employment of the GRF4-GIF1 transcriptional complex or similar booster genes could address the regeneration bottleneck currently experienced in the transformation of wheat.

Exploiting plant viruses as delivery vectors is a promising emerging strategy to obtain CRISPR/Cas-edited plants. They present a few advantages that could confront some of the challenges experienced in this study. As viruses replicate, they spread systemically to tissues throughout the plant which could result in higher expression of the CRISPR/Cas9 components and a consequent increased editing efficiency [257]. Viral genomes are also simple to engineer for the purpose of multiplex targeting. To date, viral vectors have largely been limited to delivering sgRNAs to Cas-

overexpressing plants due to the cargo capacity of the vectors not permitting the insertion of large coding sequences such as *spCas9*. This was recently illustrated in wheat. Researchers introduced an engineered *Barley stripe mosaic virus*–based sgRNA delivery vector into Cas9-transgenic wheat to produce mutant plants while bypassing tissue culture and plant regeneration [143]. Furthermore, recent studies have described the delivery into plant cells of positive- and negative-strand RNA viruses that carry both the Cas and sgRNA sequences [258,259]. Alternatively, Pausch *et al*., [260] recently revealed a hypercompact type-V CRISPR-CasΦ system comprised of a CasΦ nuclease that is roughly half the size of Cas9 or Cas12. The CRISPR-CasΦ system is also a crRNA-guided system which could potentially reduce the cargo capacity limitations implicit in viral vector-based delivery systems.

In the current study, the bombardment of mature wheat seeds with their SAMs exposed was trialed following the report published by Hamada *et al* [214]. As mentioned above, the researchers delivered plasmids expressing CRISPR/Cas9 components targeting *TaGASR7* into the SAMs of imbibed mature wheat seeds. They provided a method that makes it possible to transform cultivars recalcitrant to conventional transformation strategies and further bypassed much of the tissue culture process. Within the SAM, sub-epidermal (L2) cells have the potential to develop into germ cells [261,262]. Therefore, mutations that are introduced in the L2 cells can be inherited by the progeny of the plant. The *in planta* particle bombardment (iPB) technique was recently validated by Liu *et al*., [263] in wheat genotypes that are typically recalcitrant to traditional transformation techniques. Using the iPB technique they introduced *TaQsd1*-targeting CRISPR/Cas9 plasmids into the SAMs of mature wheat seeds and were able to retrieve a triple-recessive homozygous mutant in the T2 generation. Thus, illustrating the extension of this technique in previously recalcitrant elite wheat cultivars and its potential to generate heritable mutations in stably transformed progeny. In a recent study that is still in preprint, researchers have reported the use of CRISPR/Cas9 RNPs as a delivery strategy in imbibed mature wheat seeds [264]. This method, termed the iPB-RNP method by the authors, combined the tissue culture-evading aspects of the iPB method with the DNA-free approach of RNP delivery. Gold particles coated with CRISPR/Cas9 RNPs targeting the *TaSD1* gene, encoding for an enzyme involved in gibberellic acid synthesis, were bombarded into the SAMs of 232 mature embryos. After the bombarded seeds were grown into mature wheat plants, 16 contained mutations at the target site for an efficiency of 6.9%. With respect to the current study, both techniques combined in the iPB-RNP strategy have been performed. This could provide a strong foundation to attempt future endeavours that could validate or optimise the iPB-RNP strategy.

5.3 Conclusion

The emergence of CRISPR/Cas9 genome-editing in recent years has made an immense contribution to research in crop plants. It holds particular promise in bread wheat, where traditional and molecular breeding techniques are time-consuming and laborious. However, regulatory and public concerns over the presence of transgenes in crop plants could hamper its application. Considering this, the

development and optimisation of DNA-free methods that allow for the precise knockout of target genes without introducing transgenes could facilitate research.

In this study, we attempted to deliver CRISPR/Cas9 components into wheat for the purpose of genome-editing. Although no regenerated mutant wheat plants were produced, preliminary evidence of transient Cas9-mediated genome-editing using a DNA construct is provided. In addition, a multiplexed transient editing approach is demonstrated that could be used in a range of applications. Further, it is shown that RNP-mediated editing is an accessible and realistic method of inducing precise genomic mutations. This study utilised a variety of techniques to achieve these results, which provides insights into developing strategic pipelines while it also highlights pitfalls that are still present in tissue culture and the regeneration of wheat.

References

1. Petersen G, Seberg O, Yde M, Berthelsen K. Phylogenetic relationships of *Triticum* and *Aegilops* and evidence for the origin of the A, B, and D genomes of common wheat (*Triticum aestivum*). Mol Phylogenet Evol. 2006;39: 70–82.

2. Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, et al. 'Green revolution'genes encode mutant gibberellin response modulators. Nature. 1999;400: 256–261.

3. Hickey LT, Hafeez AN, Robinson H, Jackson SA, Leal-Bertioli SCM, Tester M, et al. Breeding crops to feed 10 billion. Nat Biotechnol. 2019;37: 744–754.

4. Rosegrant MW, Tokgoz S, Bhandary P, Msangi S. Looking ahead: scenarios for the future of food. 2015.

5. Ray DK, Mueller ND, West PC, Foley JA. Yield trends are insufficient to double global crop production by 2050. PLoS One. 2013;8: e66428.

6. Tilman D, Balzer C, Hill J, Befort BL. Global food demand and the sustainable intensification of agriculture. Proc Natl Acad Sci USA. 2011.

7. Li C, Zhang R, Meng X, Chen S, Zong Y, Lu C, et al. Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors. Nat Biotechnol. 2020;38: 875–882.

8. Char SN, Yang B. Genome editing in grass plants. aBIOTECH. 2020;1: 41–57.

9. Gürel F, Zhang Y, Sretenovic S, Qi Y. CRISPR-Cas nucleases and base editors for plant genome editing. aBIOTECH. 2020;1: 74–87.

10. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346.

11. Appels R, Eversole K, Feuillet C, Keller B, Rogers J, Stein N, et al. Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science. 2018;361.

12. Zhang Y, Li D, Zhang D, Zhao X, Cao X, Dong L, et al. Analysis of the functions of Ta GW 2 homoeologs in wheat grain weight and protein content traits. Plant J. 2018;94: 857–866.

13. Sánchez-León S, Gil-Humanes J, Ozuna C V., Giménez MJ, Sousa C, Voytas DF, et al. Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol J. 2018;16: 902–910.

14. Lv J, Yu K, Wei J, Gui H, Liu C, Liang D, et al. Generation of paternal haploids in wheat by genome editing of the centromeric histone CENH3. Nat Biotechnol. 2020;38: 1397–1401.

15. Kouranova E, Forbes K, Zhao G, Warren J, Bartels A, Wu Y, et al. CRISPRs for optimal targeting: delivery of CRISPR components as DNA, RNA, and protein into cultured cells and single-cell embryos. Hum Gene Ther. 2016;27: 464–475.

16. Shan Q, Wang Y, Li J, Gao C. Genome editing in rice and wheat using the CRISPR/Cas system. Nat Protoc. 2014;9: 2395–2410.

17. Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, et al. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Commun. 2016;7: 1–8.

18. Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, et al. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Commun. 2017;8: 1–5.

19. Collard BC, Mackill DJ. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philosophical Transactions of the Royal Society B: Biological Sciences. 2008;363: 557-72.

20. Matsuoka Y. Evolution of polyploid *triticum* wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. Plant and Cell Physiology. 2011.

21. Marcussen T, Sandve SR, Heier L, Spannagl M, Pfeifer M, Jakobsen KS, et al. Ancient hybridizations among the ancestral genomes of bread wheat. Science. 2014;345.

22. Arumuganathan K, Earle ED. Nuclear DNA content of some important plant species. Plant Mol Biol Report. 1991.

23. Bhalla PL, Sharma A, Singh MB. Enabling molecular technologies for trait improvement in wheat. Methods in Molecular Biology. 2017.

24. Adamski NM, Borrill P, Brinton J, Harrington SA, Marchal C, Bentley AR, et al. A roadmap for gene functional characterisation in crops with large genomes: Lessons from polyploid wheat. Elife. 2020;9: 1–30.

25. Garsmeur O, Droc G, Antonise R, Grimwood J, Potier B, Aitken K, et al. A mosaic monoploid reference sequence for the highly complex genome of sugarcane. Nat Commun. 2018.

26. Shrawat AK, Armstrong CL. Development and Application of Genetic Engineering for Wheat Improvement. CRC Crit Rev Plant Sci. 2018;37: 335–421.

27. Altpeter F, Springer NM, Bartley LE, Blechl AE, Brutnell TP, Citovsky V, et al. Advancing crop transformation in the era of genome editing. Plant Cell. 2016.

28. Ramírez-González RH, Borrill P, Lang D, Harrington SA, Brinton J, Venturini L, et al. The transcriptional landscape of polyploid wheat. Science. 2018.

29. null null, X. MKF, Jane R, Jaroslav D, Curtis P, Kellye E, et al. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. Science. 2014;345: 1251788.

30. Walkowiak S, Gao L, Monat C, Haberer G, Kassa MT, Brinton J, et al. Multiple wheat genomes reveal global variation in modern breeding. Nature. 2020;588: 277–283.

31. Guo W, Xin M, Wang Z, Yao Y, Hu Z, Song W, et al. Origin and adaptation to high altitude of Tibetan semi-wild wheat. Nat Commun. 2020;11: 1–12.

32. Zhu T, Wang L, Rimbert H, Rodriguez JC, Deal KR, De Oliveira R, et al. Optical maps refine the bread wheat *Triticum aestivum* cv. Chinese Spring genome assembly. Plant J. 2021;107: 303–314.

33. Sleper DA, Poehlman JM. Breeding field crops. Blackwell publishing; 2006.

34. Naruoka Y, Garland-Campbell KA, Carter AH. Genome-wide association mapping for stripe rust (*Puccinia striiformis f. sp. tritici*) in US Pacific Northwest winter wheat (*Triticum aestivum L.*). Theor Appl Genet. 2015;128: 1083–1101.

35. Koebner RMD, Summers RW. 21st century wheat breeding: plot selection or plate detection? Trends Biotechnol. 2003;21: 59–63.

36. He J, Zhao X, Laroche A, Lu Z-X, Liu H, Li Z. Genotyping-by-sequencing (GBS), an ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. Front Plant Sci. 2014;5: 484.

37. Edwards D, Batley J, Snowdon RJ. Accessing complex crop genomes with next-generation sequencing. Theor Appl Genet. 2013;126: 1–11.

38. Slatko BE, Gardner AF, Ausubel FM. Overview of next-generation sequencing technologies. Curr Protoc Mol Biol. 2018;122: e59.

39. Ahmad M. Molecular marker-assisted selection of HMW glutenin alleles related to wheat bread quality by PCR-generated DNA markers. Theor Appl Genet. 2000.

40. Wijnker E, de Jong H. Managing meiotic recombination in plant breeding. Trends Plant Sci. 2008;13: 640–646.

41. Jordan KW, Wang S, He F, Chao S, Lun Y, Paux E, et al. The genetic architecture of genome-wide recombination rate variation in allopolyploid wheat revealed by nested association mapping. Plant J. 2018;95: 1039–1054.

42. King J, Armstead IP, Donnison SI, Roberts LA, Harper JA, Skøt K, et al. Comparative analyses between Lolium/Festuca introgression lines and rice reveal the major fraction of functionally annotated gene models is located in recombination-poor/very recombination-poor regions of the genome. Genetics. 2007;177: 597–606.

43. Arrigo N, Guadagnuolo R, Lappe S, Pasche S, Parisod C, Felber F. Gene flow between wheat and wild relatives: empirical evidence from Aegilops geniculata, Ae. neglecta and Ae. triuncialis. Evol Appl. 2011;4: 685–695.

44. Cavanagh CR, Chao S, Wang S, Huang BE, Stephen S, Kiani S, et al. Genome-wide comparative diversity uncovers multiple targets of selection for improvement in hexaploid wheat landraces and cultivars. Proc Natl Acad Sci USA. 2013;110: 8057–8062.

45. Van de Wouw M, Kik C, van Hintum T, van Treuren R, Visser B. Genetic erosion in crops: concept, research results and challenges. Plant Genet Resour. 2010;8: 1–15.

46. Fichtner F, Urrea Castellanos R, Ülker B. Precision genetic modifications: a new era in molecular biology and crop improvement. Planta. 2014.

47. Sikora P, Chawade A, Larsson M, Olsson J, Olsson O. Mutagenesis as a tool in plant genetics, functional genomics, and breeding. Int J Plant Genomics. 2011;2011.

48. McCallum CM, Comai L, Greene EA, Henikoff S. Targeting induced local lesions IN genomes (TILLING)

for plant functional genomics. Plant Physiology. 2000.

49. Slade AJ, McGuire C, Loeffler D, Mullenberg J, Skinner W, Fazio G, et al. Development of high amylose wheat through TILLING. BMC Plant Biol. 2012;12.

50. Krasileva K V, Vasquez-Gross HA, Howell T, Bailey P, Paraiso F, Clissold L, et al. Uncovering hidden variation in polyploid wheat. Proc Natl Acad Sci. 2017;114: E913–E921.

51. Parry MAJ, Madgwick PJ, Bayon C, Tearall K, Hernandez-Lopez A, Baudo M, et al. Mutation discovery for crop improvement. Journal of Experimental Botany. 2009.

52. Herrera-Estrella L, Depicker A, Van Montagu M, Schell J. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature. 1983.

53. Klein TM, Sanford JC, Wolf ED, Wu R. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature. 1987.

54. An G. High Efficiency Transformation of Cultured Tobacco Cells. Plant Physiol. 1985.

55. Vasil V, Castillo AM, Fromm ME, Vasil IK. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology. 1992.

56. Becker D, Brettschneider R, Lörz H. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J. 1994.

57. Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, et al. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 1994.

58. Rasco-Gaunt S, Riley A, Cannell M, Barcelo P, Lazzeri PA. Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum L.*) varieties via particle bombardment. J Exp Bot. 2001.

59. Pellegrineschi A, Noguera LM, Skovmand B, Brito RM, Velazquez L, Salgado MM, et al. Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. Genome. 2002.

60. Wang K, Gong Q, Ye X. Recent developments and applications of genetic transformation and genome editing technologies in wheat. Theor Appl Genet. 2019.

61. Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, et al. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 1997.

62. He Y, Jones HD, Chen S, Chen XM, Wang DW, Li KX, et al. *Agrobacterium*-mediated transformation of durum wheat (*Triticum turgidum L.* var. durum cv Stewart) with improved efficiency. J Exp Bot. 2010.

63. Ishida Y, Tsunashima M, Hiei Y, Komari T. Wheat (*Triticum aestivum L.*) Transformation using immature embryos. Methods Mol Biol. 2015.

64. Richardson T, Thistleton J, Higgins TJ, Howitt C, Ayliffe M. Efficient *Agrobacterium* transformation of elite wheat germplasm without selection. Plant Cell Tissue Organ Cult. 2014.

65. Wang K, Liu H, Du L, Ye X. Generation of marker-free transgenic hexaploid wheat via an *Agrobacterium*-mediated co-transformation strategy in commercial Chinese wheat varieties. Plant Biotechnol J. 2017.

66. Curtin SJ, Voytas DF, Stupar RM. Genome Engineering of Crops with Designer Nucleases. 2012.

67. Rinaldo AR, Ayliffe M. Gene targeting and editing in crop plants : a new era of precision opportunities. 2015.

68. Harrison MM, Jenkins B V, Connor-giles KMO, Wildonger J. A CRISPR view of development. 2014; 1859–1872.

69. Puchta H, Dujon B, Hohn B. Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc Natl Acad Sci U S A. 1996.

70. Chapman JR, Taylor MRG, Boulton SJ. Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. Molecular Cell. 2012.

71. He C, Gouble A, Bourdel A, Manchev V, Poirot L, Paques F, et al. Lentiviral protein delivery of meganucleases in human cells mediates gene targeting and alleviates toxicity. Gene Ther. 2014.

72. Petersen B, Niemann H. Molecular scissors and their application in genetically modified farm animals. Transgenic Research. 2015.

73. Gao H, Smith J, Yang M, Jones S, Djukanovic V, Nicholson MG, et al. Heritable targeted mutagenesis in maize using a designed endonuclease. Plant J. 2010.

74. Puchta H, Fauser F. Synthetic nucleases for genome engineering in plants: prospects for a bright future. Plant Journal. 2014.

75. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci U S A. 1996.

76. Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, et al. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics. 2010.

77. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, et al. Breaking the code of DNA binding specificity of TAL-type III effectors. Science. 2009.

78. Haun W, Coffman A, Clasen BM, Demorest ZL, Lowy A, Ray E, et al. Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol J. 2014.

79. Wendt T, Holm PB, Starker CG, Christian M, Voytas DF, Brinch-Pedersen H, et al. TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. Plant Mol Biol. 2013.

80. Qiu J-L, Gao C, Shan Q, Liu J, Cheng X, Zhang Y, et al. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol. 2014;32: 947–951.

81. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat

Biotechnol. 2014;32: 347–350.

82. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc Natl Acad Sci U S A. 2012.

83. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, et al. An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol. 2015.

84. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, et al. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Mol Cell. 2015.

85. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012.

86. Liang Z, Zhang K, Chen K, Gao C. Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. J Genet Genomics. 2014;41: 63–68.

87. Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat Commun. 2016;7: 1–7.

88. Lawrenson T, Shorinola O, Stacey N, Li C, Østergaard L, Patron N, et al. Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. Genome Biol. 2015.

89. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res. 2014;42: 10903–10914.

90. Tang X, Zheng X, Qi Y, Zhang D, Cheng Y, Tang A, et al. A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants. Molecular Plant. 2016.

91. Upadhyay SK, Kumar J, Alok A, Tuli R. RNA-Guided Genome Editing for Target Gene Mutations in Wheat. Genes. 2013.

92. Demirci Y, Zhang B, Unver T. CRISPR/Cas9: an RNA-guided highly precise synthetic tool for plant genome editing. J Cell Physiol. 2018;233: 1844–1859.

93. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, et al. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol. 2013.

94. Wang W, Simmonds J, Pan Q, Davidson D, He F, Battal A, et al. Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. Theor Appl Genet. 2018.

95. Singh P, Arora A, Strand TA, Leffler DA, Catassi C, Green PH, et al. Global Prevalence of Celiac Disease: systematic Review and Meta-analysis. Clin Gastroenterol Hepatol. 2018.

96. Schnable PS, Springer NM. Progress Toward Understanding Heterosis in Crop Plants. Annu Rev Plant Biol. 2013.

97. Fischer S, Möhring J, Schön CC, Piepho HP, Klein D, Schipprack W, et al. Trends in genetic variance components during 30 years of hybrid maize breeding at the University of Hohenheim. Plant Breed. 2008.

98. Marulanda JJ, Mi X, Melchinger AE, Xu JL, Würschum T, Longin CFH. Optimum breeding strategies using genomic selection for hybrid breeding in wheat, maize, rye, barley, rice and triticale. Theor Appl Genet. 2016.

99. Mühleisen J, Piepho HP, Maurer HP, Longin CFH, Reif JC. Yield stability of hybrids versus lines in wheat, barley, and triticale. Theor Appl Genet. 2014.

100. Longin CFH, Gowda M, Mühleisen J, Ebmeyer E, Kazman E, Schachschneider R, et al. Hybrid wheat: quantitative genetic parameters and consequences for the design of breeding programs. Theor Appl Genet. 2013.

101. Longin CFH, Mühleisen J, Maurer HP, Zhang H, Gowda M, Reif JC. Hybrid breeding in autogamous cereals. Theoretical and Applied Genetics. 2012.

102. Singh M, Kumar M, Albertsen MC, Young JK, Cigan AM. Concurrent modifications in the three homeologs of *Ms45* gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum L.*). Plant Mol Biol. 2018;97: 371–383.

103. Okada A, Arndell T, Borisjuk N, Sharma N, Watson-Haigh NS, Tucker EJ, et al. CRISPR/Cas9-mediated knockout of *Ms1* enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. Plant Biotechnol J. 2019;17: 1905–1913.

104. Zhong Y, Liu C, Qi X, Jiao Y, Wang D, Wang Y, et al. Mutation of *ZmDMP* enhances haploid induction in maize. Nature Plants. 2019.

105. Yao L, Zhang Y, Liu C, Liu Y, Wang Y, Liang D, et al. *OsMATL* mutation induces haploid seed formation in indica rice. Nat Plants. 2018.

106. Liu C, Zhong Y, Qi X, Chen M, Liu Z, Chen C, et al. Extension of the in vivo haploid induction system from diploid maize to hexaploid wheat. Plant Biotechnol J. 2020;18: 316–318.

107. Kelliher T, Starr D, Richbourg L, Chintamanani S, Delzer B, Nuccio ML, et al. MATRILINEAL, a sperm-specific phospholipase, triggers maize haploid induction. Nature. 2017.

108. Wang C, Liu Q, Shen Y, Hua Y, Wang J, Lin J, et al. Clonal seeds in hybrid rice using CRISPR/Cas9. bioRxiv. 2018; 496042.

109. Liu H, Wang K, Jia Z, Gong Q, Lin Z, Du L, et al. Efficient induction of haploid plants in wheat by editing of *TaMTL* using an optimized *Agrobacterium*-mediated CRISPR system. J Exp Bot. 2020;71: 1337–1349.

110. Lin J, Mercier R, Wu M, Wang C, Wang K, Liu Q, et al. Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes. Nat Biotechnol. 2019.

111. Howells RM, Craze M, Bowden S, Wallington EJ. Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. BMC Plant Biol. 2018;18: 1–11.

112. Trick H, He F, Chao S, Akhunov E, Akhunova A, Pan Q, et al. Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. Cris J. 2018;1: 65–74.

113. Zhang S, Zhang R, Song G, Gao J, Li W, Han X, et al. Targeted mutagenesis using the *Agrobacterium tumefaciens*-mediated CRISPR-Cas9 system in common wheat. BMC Plant Biol. 2018.

114. Wang W, Pan Q, Tian B, He F, Chen Y, Bai G, et al. Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat. Plant J. 2019.

115. Zhang Z, Hua L, Gupta A, Tricoli D, Edwards KJ, Yang B, et al. Development of an *Agrobacterium*-delivered CRISPR/Cas9 system for wheat genome editing. Plant Biotechnol J. 2019;17: 1623–1635.

116. Abe F, Haque E, Hisano H, Tanaka T, Kamiya Y, Mikami M, et al. Genome-Edited Triple-Recessive Mutation Alters Seed Dormancy in Wheat. Cell Rep. 2019;28: 1362-1369.e4.

117. Brauer EK, Balcerzak M, Rocheleau H, Leung W, Schernthaner J, Subramaniam R, et al. Genome Editing of a Deoxynivalenol-Induced Transcription Factor Confers Resistance to Fusarium graminearum in Wheat. Mol Plant-Microbe Interact. 2020;33: 553–560.

118. Li J, Wang Z, He G, Ma L, Deng XW. CRISPR/Cas9-mediated disruption of *TaNP1* genes results in complete male sterility in bread wheat. J Genet Genomics. 2020;47: 263–272.

119. Tang H, Liu H, Zhou Y, Liu H, Du L, Wang K, et al. Fertility recovery of wheat male sterility controlled by *Ms2* using CRISPR/Cas9. Plant Biotechnol J. 2021;19: 224–226.

120. Liu H, Wang K, Tang H, Gong Q, Du L, Pei X, et al. CRISPR/Cas9 editing of wheat *TaQ* genes alters spike morphogenesis and grain threshability. J Genet Genomics. 2020;47: 563–575.

121. Li J, Jiao G, Sun Y, Chen J, Zhong Y, Yan L, et al. Modification of starch composition, structure and properties through editing of *TaSBEIIa* in both winter and spring wheat varieties by CRISPR/Cas9. Plant Biotechnol J. 2021;19: 937–951.

122. Zhang J, Zhang H, Li S, Li J, Yan L, Xia L. Increasing yield potential through manipulating of an *ARE1* ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. J Integr Plant Biol. 2021;63: 1649–1663.

123. Ibrahim S, Saleem B, Rehman N, Zafar SA, Naeem MK, Khan MR. CRISPR/Cas9 mediated disruption of *Inositol Pentakisphosphate 2-Kinase 1 (TaIPK1)* reduces phytic acid and improves iron and zinc accumulation in wheat grains. J Adv Res. 2021;1.

124. Raffan S, Sparks C, Huttly A, Hyde L, Martignago D, Mead A, et al. Wheat with greatly reduced accumulation of free asparagine in the grain, produced by CRISPR/Cas9 editing of asparagine synthetase gene *TaASN2*. Plant Biotechnol J. 2021;19: 1602–1613.

125. Zhang S, Zhang R, Gao J, Song G, Li J, Li W, et al. CRISPR/Cas9-mediated genome editing for wheat grain quality improvement. Plant Biotechnol J. 2021;19: 1684–1686.

126. Curtis TY, Raffan S, Wan Y, King R, Gonzalez-Uriarte A, Halford NG. Contrasting gene expression patterns in grain of high and low asparagine wheat genotypes in response to sulphur supply. BMC Genomics. 2019;20: 1–18.

127. Woo JW, Kim J, Kwon S Il, Corvalán C, Cho SW, Kim H, et al. DNA-free genome editing in plants with

preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol. 2015.

128. Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, et al. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Commun. 2017;8: 6–10.

129. Mahas A, Ali Z, Tashkandi M, Mahfouz MM. Virus-mediated genome editing in plants using the CRISPR/Cas9 system. Plant Genome Editing with CRISPR Systems. Springer; 2019. pp. 311–326.

130. Hu J, Li S, Li Z, Li H, Song W, Zhao H, et al. A barley stripe mosaic virus-based guide RNA delivery system for targeted mutagenesis in wheat and maize. Mol Plant Pathol. 2019;20: 1463–1474.

131. Ali Z, Abul-Faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, et al. Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol Plant. 2015;8: 1288–1291.

132. Cody WB, Scholthof HB, Mirkov TE. Multiplexed gene editing and protein overexpression using a tobacco mosaic virus viral vector. Plant Physiol. 2017;175: 23–35.

133. Jiang N, Zhang C, Liu J, Guo Z, Zhang Z, Han C, et al. Development of Beet necrotic yellow vein virus-based vectors for multiple-gene expression and guide RNA delivery in plant genome editing. Plant Biotechnol J. 2019;17: 1302–1315.

134. Ali Z, Eid A, Ali S, Mahfouz MM. Pea early-browning virus-mediated genome editing via the CRISPR/Cas9 system in *Nicotiana benthamiana* and *Arabidopsis*. Virus Res. 2018;244: 333–337.

135. Yin K, Han T, Liu G, Chen T, Wang Y, Yu AYL, et al. A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci Rep. 2015;5: 1–10.

136. Dahan-Meir T, Filler-Hayut S, Melamed-Bessudo C, Bocobza S, Czosnek H, Aharoni A, et al. Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. Plant J. 2018;95: 5–16.

137. Butler NM, Baltes NJ, Voytas DF, Douches DS. Geminivirus-mediated genome editing in potato (Solanum tuberosum L.) using sequence-specific nucleases. Front Plant Sci. 2016;7: 1045.

138. Wang M, Lu Y, Botella JR, Mao Y, Hua K, Zhu J. Gene targeting by homology-directed repair in rice using a geminivirus-based CRISPR/Cas9 system. Mol Plant. 2017;10: 1007–1010.

139. Gil-Humanes J, Wang Y, Liang Z, Shan Q, Ozuna C V., Sánchez-León S, et al. High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. Plant J. 2017;89: 1251–1262.

140. Senthil-Kumar M, Mysore KS. Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. Plant Biotechnol J. 2011;9: 797–806.

141. Shen W, Hohn B. Amplification and expression of the β-glucuronidase gene in maize plants by vectors based on maize streak virus. Plant J. 1994;5: 227–236.

142. Shen W-H, Hohn B. Mutational analysis of the small intergenic region of maize streak virus. Virology. 1991;183: 721–730.

143. Li T, Hu J, Sun Y, Li B, Zhang D, Li W, et al. Highly efficient heritable genome editing in wheat using an RNA virus and bypassing tissue culture. Mol Plant. 2021;14: 1787–1798.

144. Ellison EE, Nagalakshmi U, Gamo ME, Huang P, Dinesh-Kumar S, Voytas DF. Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. Nat plants. 2020;6: 620–624.

145. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS, et al. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front Plant Sci. 2016;7: 1–9.

146. Murovec J, Guček K, Bohanec B, Avbelj M, Jerala R. DNA-Free Genome Editing of *Brassica oleracea* and *B. rapa* Protoplasts Using CRISPR-Cas9 Ribonucleoprotein Complexes. Front Plant Sci. 2018;9: 1–9.

147. Andersson M, Turesson H, Olsson N, Fält AS, Ohlsson P, Gonzalez MN, et al. Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol Plant. 2018;164: 378–384.

148. Banakar R, Schubert M, Collingwood M, Vakulskas C, Eggenberger AL, Wang K. Comparison of CRISPR-Cas9/Cas12a Ribonucleoprotein Complexes for Genome Editing Efficiency in the Rice Phytoene Desaturase (OsPDS) Gene. Rice. 2020;13.

149. Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat Commun. 2016.

150. Banakar R, Eggenberger AL, Lee K, Wright DA, Murugan K, Zarecor S, et al. High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice. Sci Rep. 2019;9: 1–13.

151. Toda E, Koiso N, Takebayashi A, Ichikawa M, Kiba T, Osakabe K, et al. An efficient DNA- and selectable-marker-free genome-editing system using zygotes in rice. Nat Plants. 2019;5: 363–368.

152. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, et al. Targeted genome modification of crop plants using a CRISPR-Cas system. Nature Biotechnology. 2013.

153. Zhang Y, Bai Y, Wu G, Zou S, Chen Y, Gao C, et al. Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. Plant J. 2017;91: 714–724.

154. Chakrabarti AM, Henser-Brownhill T, Monserrat J, Poetsch AR, Luscombe NM, Scaffidi P. Target-specific precision of CRISPR-mediated genome editing. Mol Cell. 2019;73: 699–713.

155. Sentmanat MF, Peters ST, Florian CP, Connelly JP, Pruett-Miller SM. A Survey of Validation Strategies for CRISPR-Cas9 Editing. Sci Rep. 2018;8: 1–8.

156. Yang Z, Steentoft C, Hauge C, Hansen L, Thomsen AL, Niola F, et al. Fast and sensitive detection of indels induced by precise gene targeting. Nucleic Acids Res. 2015;43: e59–e59.

157. Brinkman EK, Chen T, Amendola M, Van Steensel B. Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. 2014;42: 1–8.

158. Hsiau T, Conant D, Rossi N, Maures T, Waite K, Yang J, et al. Inference of CRISPR Edits from Sanger

Trace Data. bioRxiv. 2018; 1–17.

159. Bennett EP, Petersen BL, Johansen IE, Niu Y, Yang Z, Chamberlain CA, et al. INDEL detection, the “Achilles heel” of precise genome editing: A survey of methods for accurate profiling of gene editing induced indels. Nucleic Acids Res. 2021;48: 11958–11981.

160. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol. 2013;31: 233–239.

161. Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, et al. The CRISPR/C as9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J. 2014;12: 797–807.

162. Liu H, Ding Y, Zhou Y, Jin W, Xie K, Chen L-L. CRISPR-P 2.0: an improved CRISPR-Cas9 tool for genome editing in plants. Mol Plant. 2017;10: 530–532.

163. Cram D, Kulkarni M, Buchwaldt M, Rajagopalan N, Bhowmik P, Rozwadowski K, et al. WheatCRISPR: a web-based guide RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. BMC Plant Biol. 2019;19: 1–8.

164. Xiao A, Cheng Z, Kong L, Zhu Z, Lin S, Gao G, et al. CasOT: a genome-wide Cas9/gRNA off-target searching tool. Bioinformatics. 2014;30: 1180–1182.

165. Bae S, Park J, Kim J-S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics. 2014;30: 1473–1475.

166. Biswas S, Li R, Hong J, Zhao X, Yuan Z, Zhang D, et al. Effective identification of CRISPR/Cas9-induced and naturally occurred mutations in rice using a multiplex ligation-dependent probe amplification-based method. Theor Appl Genet. 2020;133.

167. Zischewski J, Fischer R, Bortesi L. Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. Biotechnol Adv. 2017;35: 95–104.

168. Modrzejewski D, Hartung F, Sprink T, Krause D, Kohl C, Wilhelm R. What is the available evidence for the range of applications of genome - editing as a new tool for plant trait modification and the potential occurrence of associated off - target effects : a systematic map. Environ Evid. 2019; 1–33.

169. Hong J, Shi Q, Biswas S, Jiang SC, Shi J. Moving genome edited crops forward from the laboratory bench to the kitchen table. Food Control. 2021;122: 107790.

170. Shiferaw B, Smale M, Braun HJ, Duveiller E, Reynolds M, Muricho G. Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. Food Secur. 2013.

171. Langridge P. Wheat genomics and the ambitious targets for future wheat production. Genome. 2013.

172. Arora L, Narula A. Gene editing and crop improvement using CRISPR-Cas9 system. Front Plant Sci. 2017;8: 1932.

173. Malzahn A, Lowder L, Qi Y. Plant genome editing with TALEN and CRISPR. Cell Biosci. 2017;7: 1–18.

174. Shi T-Q, Liu G-N, Ji R-Y, Shi K, Song P, Ren L-J, et al. CRISPR/Cas9-based genome editing of the filamentous fungi: the state of the art. Appl Microbiol Biotechnol. 2017;101: 7435–7443.

175. Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. Nucleic Acids Res. 2013;41: e188–e188.

176. Peng A, Chen S, Lei T, Xu L, He Y, Wu L, et al. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene Cs LOB 1 promoter in citrus. Plant Biotechnol J. 2017;15: 1509–1519.

177. Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv. 2015;33: 41–52.

178. Jaganathan D, Ramasamy K, Sellamuthu G, Jayabalan S, Venkataraman G. CRISPR for Crop Improvement: An Update Review. Front Plant Sci. 2018.

179. Langner T, Kamoun S, Belhaj K. CRISPR crops: plant genome editing toward disease resistance. Annu Rev Phytopathol. 2018;56: 479–512.

180. Rogowsky PM. CRISPR-Cas Technology in Plant Science. Potato Res. 2017;60: 353–360.

181. Parsons JD, MacKay J. The theory and applications of CRISPR in plant and tree improvement. CAB Rev. 2018;13: 1–12.

182. Zeng J, He G, Wang X, Yang G, Wang Q, Li M, et al. The lycopene β-cyclase plays a significant role in provitamin A biosynthesis in wheat endosperm. BMC Plant Biol. 2015;15: 1–14.

183. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980;8: 4321–4326.

184. Hirschberg J. Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol. 2001;4: 210–218.

185. Yang Z, Bai Z, Li X, Wang P, Wu Q, Yang L, et al. SNP identification and allelic-specific PCR markers development for TaGW2, a gene linked to wheat kernel weight. Theor Appl Genet. 2012;125: 1057–1068.

186. Liang Z, Chen K, Zhang Y, Liu J, Yin K, Qiu JL, et al. Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in vitro transcripts or ribonucleoproteins. Nat Protoc. 2018;13: 413–430.

187. Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, Cheng QW, et al. Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnol J. 2018;16: 1295–1310.

188. Travella S, Klimm TE, Keller B. RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat. Plant Physiol. 2006;142: 6–20.

189. Yang J, Zhang T-Y, Liao Q-S, He L, Li J, Zhang H-M, et al. Chinese Wheat Mosaic Virus-Induced Gene Silencing in Monocots and Dicots at Low Temperature. Front Plant Sci. 2018;9: 1627.

190. Kim D, Hager M, Brant E, Budak H. Efficient genome editing in wheat using Cas9 and Cpf1 (AsCpf1 and LbCpf1) nucleases. Funct Integr Genomics. 2021;21: 355–366.

191. Protoplasts M, Ribeiro R, Sant A, Caprestano CA, Nodari RO, Agapito-tenfen SZ. PEG-Delivered CRISPR-Cas9 Ribonucleoproteins System for Gene-Editing Screening of Maize Protoplasts. 2020; 1–14.

192. Brandt KM, Gunn H, Moretti N, Zemetra RS. A Streamlined Protocol for Wheat (*Triticum aestivum*) Protoplast Isolation and Transformation With CRISPR-Cas Ribonucleoprotein Complexes. Front Plant Sci. 2020;11: 1–14.

193. Fan Y, Xin S, Dai X, Yang X, Huang H, Hua Y. Industrial Crops & Products E ffi cient genome editing of rubber tree (*hevea brasiliensis*) protoplasts using CRISPR / Cas9 ribonucleoproteins. Ind Crop Prod. 2020;146: 112146.

194. Janga MR, Campbell LAM, Rathore KS. CRISPR/Cas9-mediated targeted mutagenesis in upland cotton (Gossypium hirsutum L.). Plant Mol Biol. 2017;94: 349–360.

195. Frank MH, Chitwood DH. Plant chimeras: The good, the bad, and the 'Bizzaria.' Dev Biol. 2016;419: 41–53.

196. Chen GQ. Effective reduction of chimeric tissue in transgenics for the stable genetic transformation of Lesquerella fendleri. HortScience. 2011;46: 86–90.

197. Jordan MC. Green fluorescent protein as a visual marker for wheat transformation. Plant Cell Rep. 2000;19: 1069–1075.

198. Sparks CA, Jones HD. Transformation of wheat by biolistics. Transgenic crops of the world. Springer; 2004. pp. 19–34.

199. Kumar R, Mamrutha HM, Kaur A, Venkatesh K, Grewal A, Kumar R, et al. Development of an efficient and reproducible regeneration system in wheat (*Triticum aestivum L*.). Physiol Mol Biol Plants. 2017;23: 945–954.

200. Sears RG, Deckard EL. Tissue Culture Variability in Wheat: Callus Induction and Plant Regeneration 1. Crop Sci. 1982;22: 546–550.

201. Chowdhury MKU, Vasil V, Vasil IK. Molecular analysis of plants regenerated from embryogenic cultures of wheat (*Triticum aestivum L*.). Theor Appl Genet. 1994;87: 821–828.

202. Sparks CA, Jones HD. Biolistics transformation of wheat. Transgenic Wheat, Barley and Oats. Springer; 2009. pp. 71–92.

203. Fu X, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, et al. Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res. 2000;9: 11–19.

204. Jouanin A, Schaart JG, Boyd LA, Cockram J, Leigh FJ, Bates R, et al. Outlook for coeliac disease patients : towards bread wheat with hypoimmunogenic gluten by gene editing of α - and γ -gliadin gene

families. 2019; 1–16.

205. Baltes NJ, Voytas DF. Enabling plant synthetic biology through genome engineering. Trends Biotechnol. 2015;33: 120–131.

206. Xie K, Minkenberg B, Yang Y. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci. 2015;112: 3570–3575.

207. Tang X, Zhong Z, Ren Q, Liu B, Zhang Y. A Single transcript CRISPR-cas9 system for multiplex genome editing in plants. Plant Genome Editing with CRISPR Systems. Springer; 2019. pp. 75–82.

208. Camerlengo F, Frittelli A, Sparks C, Doherty A, Martignago D, Larré C, et al. CRISPR-Cas9 Multiplex Editing of the α-Amylase/Trypsin Inhibitor Genes to Reduce Allergen Proteins in Durum Wheat. Front Sustain Food Syst. 2020;4.

209. Luo J, Li S, Xu J, Yan L, Ma Y, Xia L. Pyramiding favorable alleles in an elite wheat variety in one generation by CRISPR-Cas9-mediated multiplex gene editing. Mol Plant. 2021;14: 847–850.

210. Henggeler JC, Veríssimo M, Ramos F. Non-coeliac gluten sensitivity: A review of the literature. Trends Food Sci Technol. 2017;66: 84–92.

211. Belanto JJ, Gil-Humanes J, Konečná E, Curtin SJ, Voytas DF, Cermak T, et al. A multi-purpose toolkit to enable advanced genome engineering in plants. Plant Cell. 2017;29: tpc.00922.2016.

212. Éva C, Csóti L, Tamás L. *Agrobacterium*-mediated barley transformation. Acta Biol Szeged. 2008;52: 49–51.

213. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15: 473–497.

214. Hamada H, Linghu Q, Nagira Y, Miki R, Taoka N, Imai R. An in planta biolistic method for stable wheat transformation. Sci Rep. 2017;7: 2–9.

215. Tian B, Navia-Urrutia M, Chen Y, Brungardt J, Trick HN. Biolistic transformation of wheat. Methods Mol Biol. 2019;1864: 117–130.

216. Sparks CA, Doherty A. Genetic transformation of common wheat (*Triticum aestivum L.*) using biolistics. 2020.

217. Hayta S, Smedley MA, Clarke M, Forner M, Harwood WA. An Efficient *Agrobacterium*-Mediated Transformation Protocol for Hexaploid and Tetraploid Wheat. Curr Protoc. 2021;1: 1–15.

218. Smedley M, Sparrow P, Harwood W. pBRACT-crop transformation vectors. Asp Appl Biol. 2011.

219. Christensen AH, Quail PH. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. 1996;5: 213–218.

220. Cao J, Wu L, Zhang S-M, Lu M, Cheung WKC, Cai W, et al. An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting. Nucleic Acids Res. 2016;44: e149–e149.

221. Char SN, Neelakandan AK, Nahampun H, Frame B, Main M, Spalding MH, et al. An *Agrobacterium*-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. Plant Biotechnol J. 2017;15: 257–268.

222. Ordon J, Gantner J, Kemna J, Schwalgun L, Reschke M, Streubel J, et al. Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit. Plant J. 2017;89: 155–168.

223. Gao Y, Zhao Y. Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. J Integr Plant Biol. 2014;56: 343–349.

224. Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, et al. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol. 2014;32: 569–576.

225. Yan W, Chen D, Kaufmann K. Efficient multiplex mutagenesis by RNA-guided Cas9 and its use in the characterization of regulatory elements in the AGAMOUS gene. Plant Methods. 2016;12: 1–9.

226. Hayta S, Smedley MA, Clarke M, Forner M, Harwood WA. An Efficient *Agrobacterium*-mediated Transformation Protocol for Hexaploid and Tetraploid Wheat. 2021; 1–15.

227. Hayta S, Smedley MA, Demir SU, Blundell R, Hinchliffe A, Atkinson N, et al. An efficient and reproducible *Agrobacterium*-mediated transformation method for hexaploid wheat (*Triticum aestivum L*.). Plant Methods. 2019;15: 1–15.

228. Jones TJ. Maize tissue culture and transformation: the first 20 years. Molecular Genetic Approaches to Maize Improvement. Springer; 2009. pp. 7–27.

229. Hiei Y, Ishida Y, Komari T. Progress of cereal transformation technology mediated by *Agrobacterium tumefaciens*. Front Plant Sci. 2014;5: 628.

230. Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, et al. Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed. 2005;15: 305–327.

231. Gordon-Kamm W, Barone P, Svitashev S, Sander JD, Kumar S, Jones T. Strategies for CRISPR/Cas9-mediated genome editing: from delivery to production of modified plants. Genome Ed Precis Crop Breed. 2021; 195–230.

232. Mehravar M, Shirazi A, Nazari M, Banan M. Mosaicism in CRISPR/Cas9-mediated genome editing. Dev Biol. 2019;445: 156–162.

233. Risacher T, Craze M, Bowden S, Paul W, Barsby T. Highly efficient *Agrobacterium*-mediated transformation of wheat via in planta inoculation. Transgenic Wheat, Barley and Oats. Springer; 2009. pp. 115–124.

234. Zale JM, Agarwal S, Loar S, Steber CM. Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. Plant Cell Rep. 2009;28: 903–913.

235. Goldberg RB, Beals TP, Sanders PM. Anther development: basic principles and practical applications. Plant Cell. 1993;5: 1217.

236. Reiser L, Fischer RL. The ovule and the embryo sac. Plant Cell. 1993;5: 1291.

237. Jensen KT, Fløe L, Petersen TS, Huang J, Xu F, Bolund L, et al. Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. FEBS Lett. 2017;591: 1892–1901.

238. Naim F, Shand K, Hayashi S, O'Brien M, McGree J, Johnson AAT, et al. Are the current gRNA ranking prediction algorithms useful for genome editing in plants? PLoS One. 2020;15: 1–12.

239. Milner MJ, Craze M, Hope MS, Wallington EJ. Turning Up the Temperature on CRISPR: Increased Temperature Can Improve the Editing Efficiency of Wheat Using CRISPR/Cas9. Front Plant Sci. 2020;11: 1–9.

240. He C, Liu H, Chen D, Xie WZ, Wang M, Li Y, et al. CRISPR-Cereal: a guide RNA design tool integrating regulome and genomic variation for wheat, maize and rice. Plant Biotechnol J. 2021; 2141–2143.

241. Zhang Y, Malzahn AA, Sretenovic S, Qi Y. The emerging and uncultivated potential of CRISPR technology in plant science. Nat Plants. 2019;5: 778–794.

242. Li J, Xu R, Qin R, Liu X, Kong F, Wei P. Genome editing mediated by SpCas9 variants with broad non-canonical PAM compatibility in plants. Mol Plant. 2021;14: 352–360.

243. Zhang Y, Zhang Q, Chen Q-J. *Agrobacterium*-mediated delivery of CRISPR/Cas reagents for genome editing in plants enters an era of ternary vector systems. Sci China Life Sci. 2020;63: 1491–1498.

244. Gao C. Genome engineering for crop improvement and future agriculture. Cell. 2021;184: 1621–1635.

245. Lowe K, La Rota M, Hoerster G, Hastings C, Wang N, Chamberlin M, et al. Rapid genotype "independent" Zea mays L.(maize) transformation via direct somatic embryogenesis. Vitr Cell Dev Biol. 2018;54: 240–252.

246. Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho M-J, et al. Morphogenic regulators Baby boom and Wuschel improve monocot transformation. Plant Cell. 2016;28: 1998–2015.

247. Mookkan M, Nelson-Vasilchik K, Hague J, Zhang ZJ, Kausch AP. Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators BABY BOOM and WUSCHEL2. Plant Cell Rep. 2017;36: 1477–1491.

248. Ke W, Shi L, Liang X, Zhao P, Wang W, Liu J, et al. The gene TaCB1 overcomes genotype dependency in wheat genetic transformation. 2021.

249. Kim JH. Biological roles and an evolutionary sketch of the GRF-GIF transcriptional complex in plants. BMB Rep. 2019;52: 227.

250. Liebsch D, Palatnik JF. MicroRNA miR396, GRF transcription factors and GIF co-regulators: a conserved plant growth regulatory module with potential for breeding and biotechnology. Curr Opin Plant Biol. 2020;53: 31–42.

251. Omidbakhshfard MA, Proost S, Fujikura U, Mueller-Roeber B. Growth-regulating factors (GRFs): a small transcription factor family with important functions in plant biology. Mol Plant. 2015;8: 998–1010.

252. Ma K, Liu Y-G. DELLA-GRF4-mediated coordination of growth and nitrogen metabolism paves the way for a new green revolution. Sci China Life Sci. 2018;61: 1130–1131.

253. Hu S, Yang H, Gao H, Yan J, Xie D. Control of seed size by jasmonate. Sci China Life Sci. 2021;64: 1215–1226.

254. Shimano S, Hibara K, Furuya T, Arimura S, Tsukaya H, Itoh J-I. Conserved functional control, but distinct regulation, of cell proliferation in rice and *Arabidopsis* leaves revealed by comparative analysis of GRF-INTERACTING FACTOR 1 orthologs. Development. 2018;145: dev159624.

255. Debernardi JM, Tricoli DM, Ercoli MF, Hayta S, Ronald P, Palatnik JF, et al. A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat Biotechnol. 2020;38: 1274–1279.

256. Qiu F, Xing S, Xue C, Liu J, Chen K, Chai T, et al. Transient expression of a TaGRF4-TaGIF1 complex stimulates wheat regeneration and improves genome editing. Sci China Life Sci. 2021; 1–8.

257. Varanda CMR, Félix MDR, Campos MD, Patanita M, Materatski P. Plant viruses: From targets to tools for CRISPR. Viruses. 2021;13: 1–19.

258. Ariga H, Toki S, Ishibashi K. Potato virus X vector-mediated DNA-free genome editing in plants. Plant Cell Physiol. 2020;61: 1946–1953.

259. Ma X, Zhang X, Liu H, Li Z. Highly efficient DNA-free plant genome editing using virally delivered CRISPR–Cas9. Nat Plants. 2020;6: 773–779.

260. Pausch P, Al-Shayeb B, Bisom-Rapp E, Tsuchida CA, Li Z, Cress BF, et al. CRISPR-CasΦ from huge phages is a hypercompact genome editor. Science. 2020;369: 333–337.

261. McDaniel CN, Poethig RS. Cell-lineage patterns in the shoot apical meristem of the germinating maize embryo. Planta. 1988;175: 13–22.

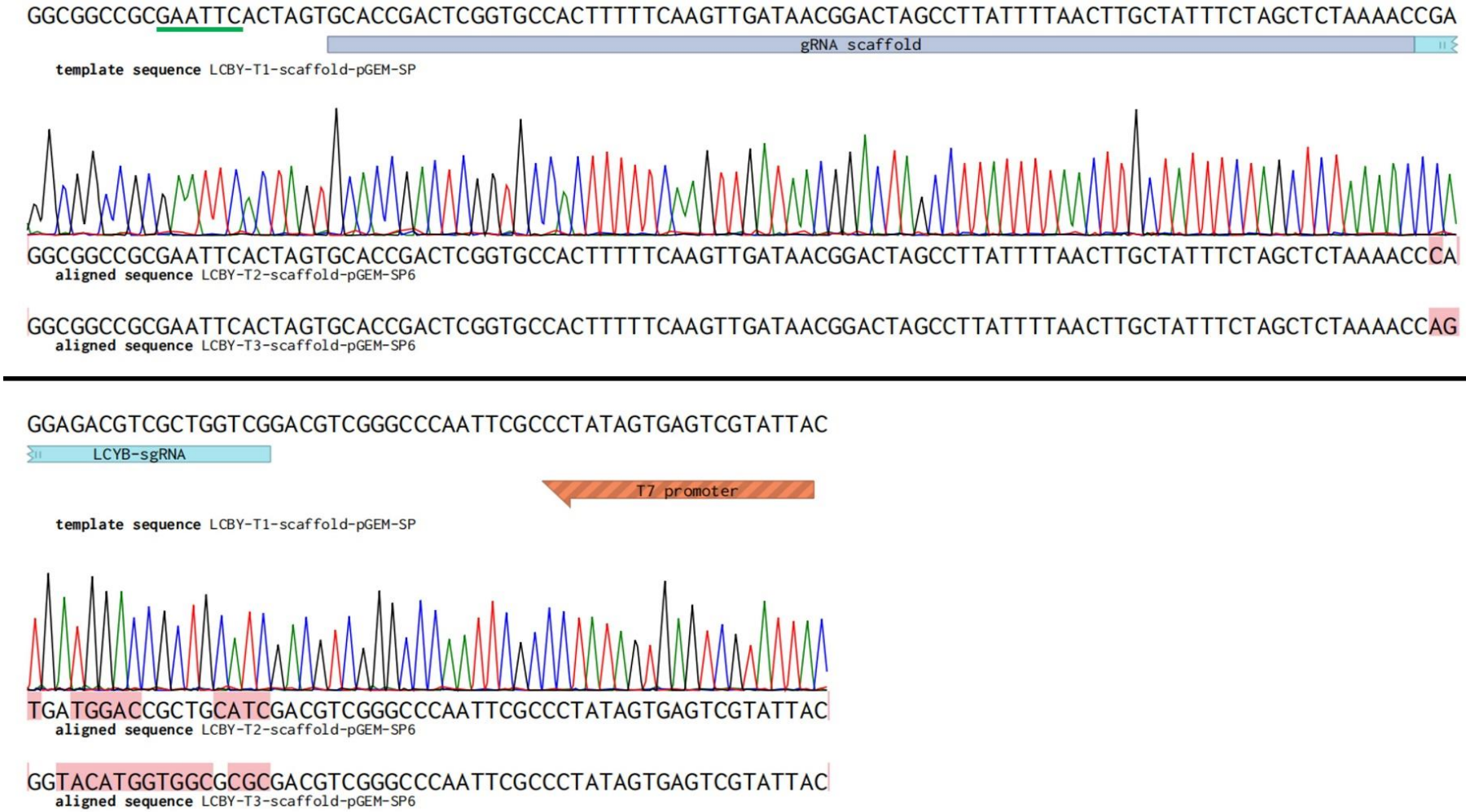
262. Irish VF, Sussex IM. A fate map of the *Arabidopsis* embryonic shoot apical meristem. Development. 1992;115: 745–753.

263. Liu Y, Luo W, Linghu Q, Abe F, Hisano H, Sato K, et al. In planta Genome Editing in Commercial Wheat Varieties. Front Plant Sci. 2021;12: 1–8.

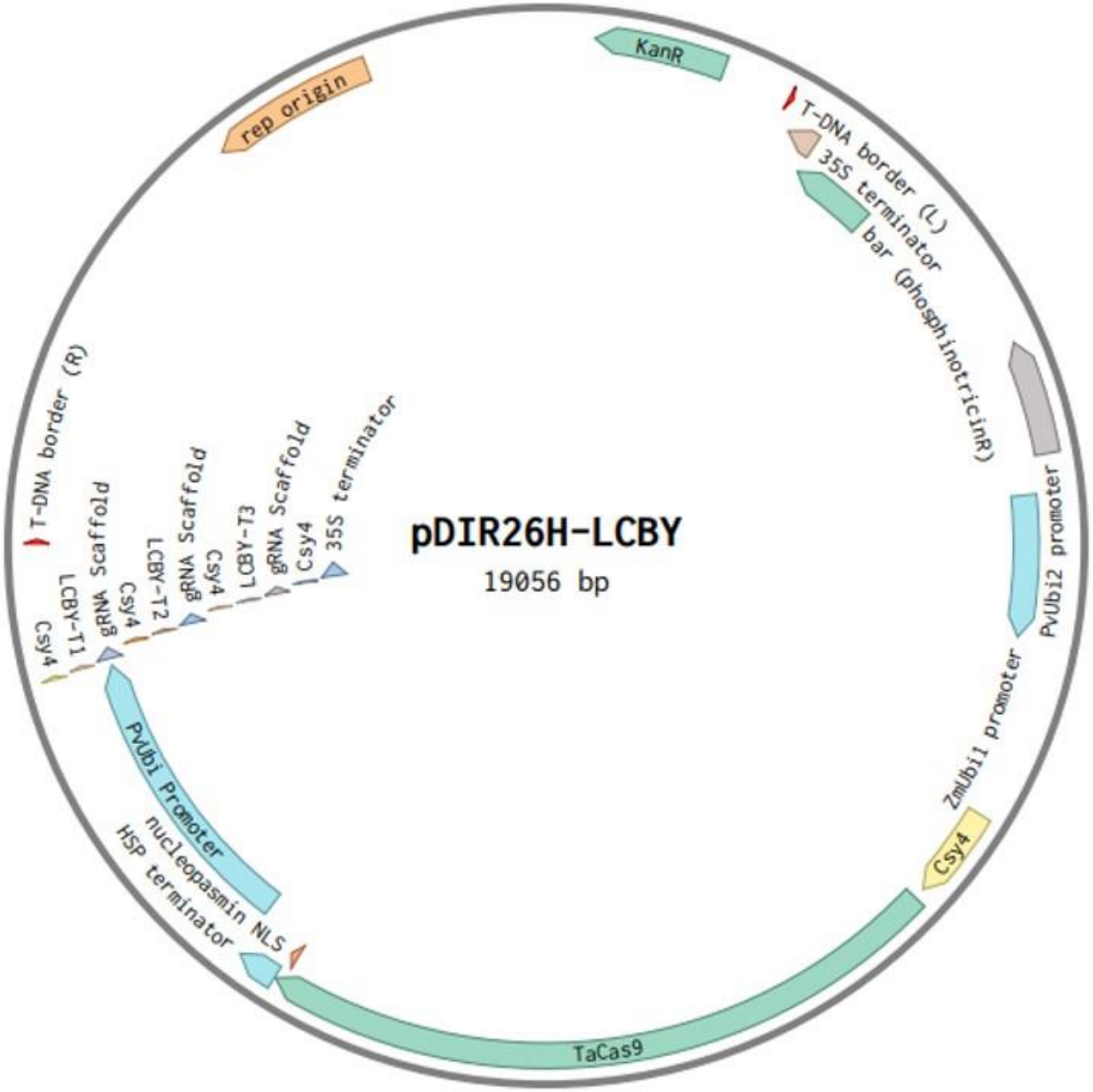
264. Kumagai Y, Liu Y, Hamada H, Luo W, Zhu J, Kuroki M, et al. In planta genome editing with CRISPR/Cas9 ribonucleoproteins. 2021.

Appendix - Supplementary material

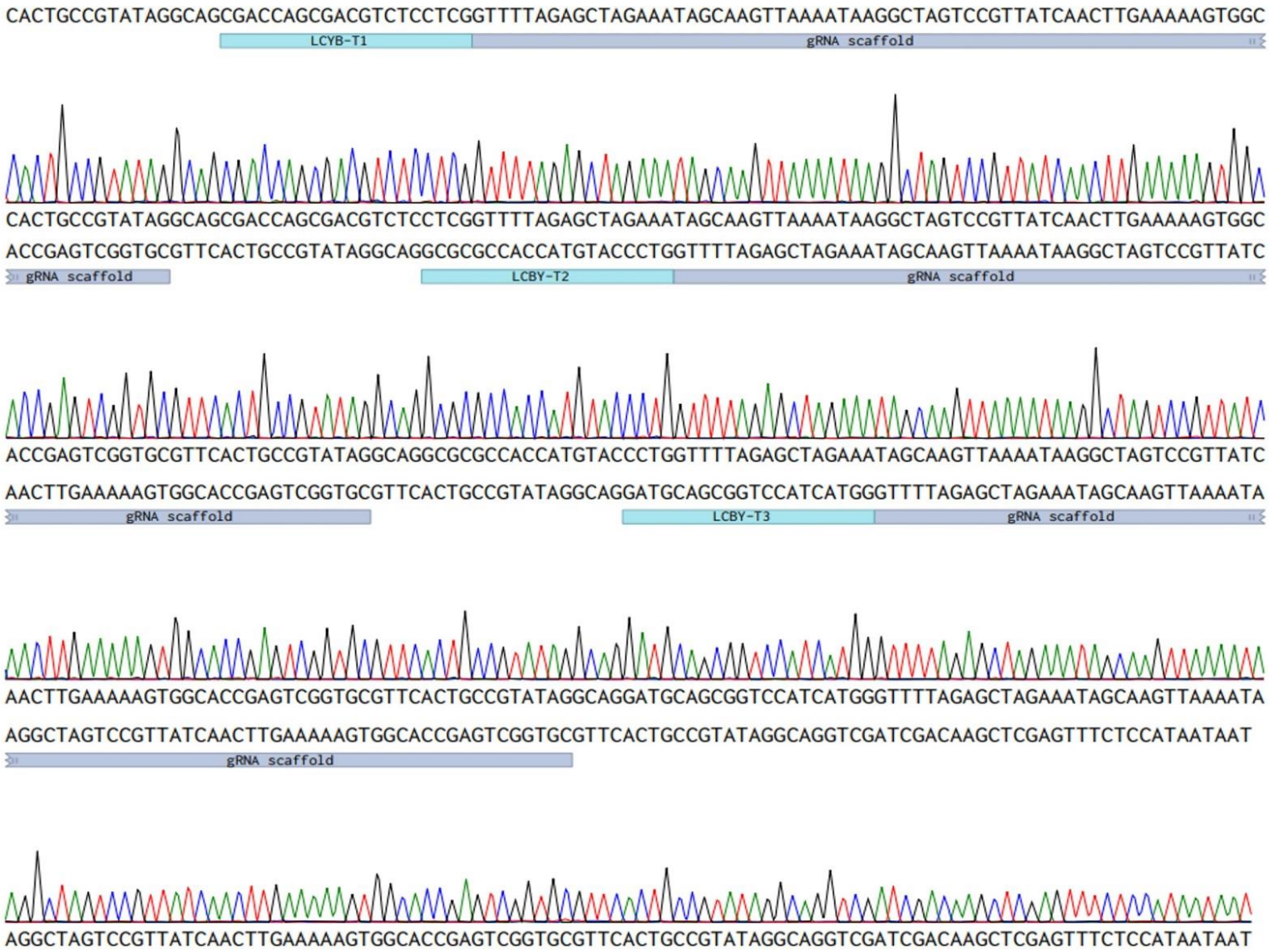
Supplementary Figure 1. TaLCYB *A, B, and D homeolog sequences spanning the three target sites targeted in this study. First row = A homeolog; Second row = B homeolog; Third row = D homeolog. Mismatches are shown in red. Primers that amplify the region containing the three target sites flank the sequence in orange. Target sequences denoted in blue bars.*



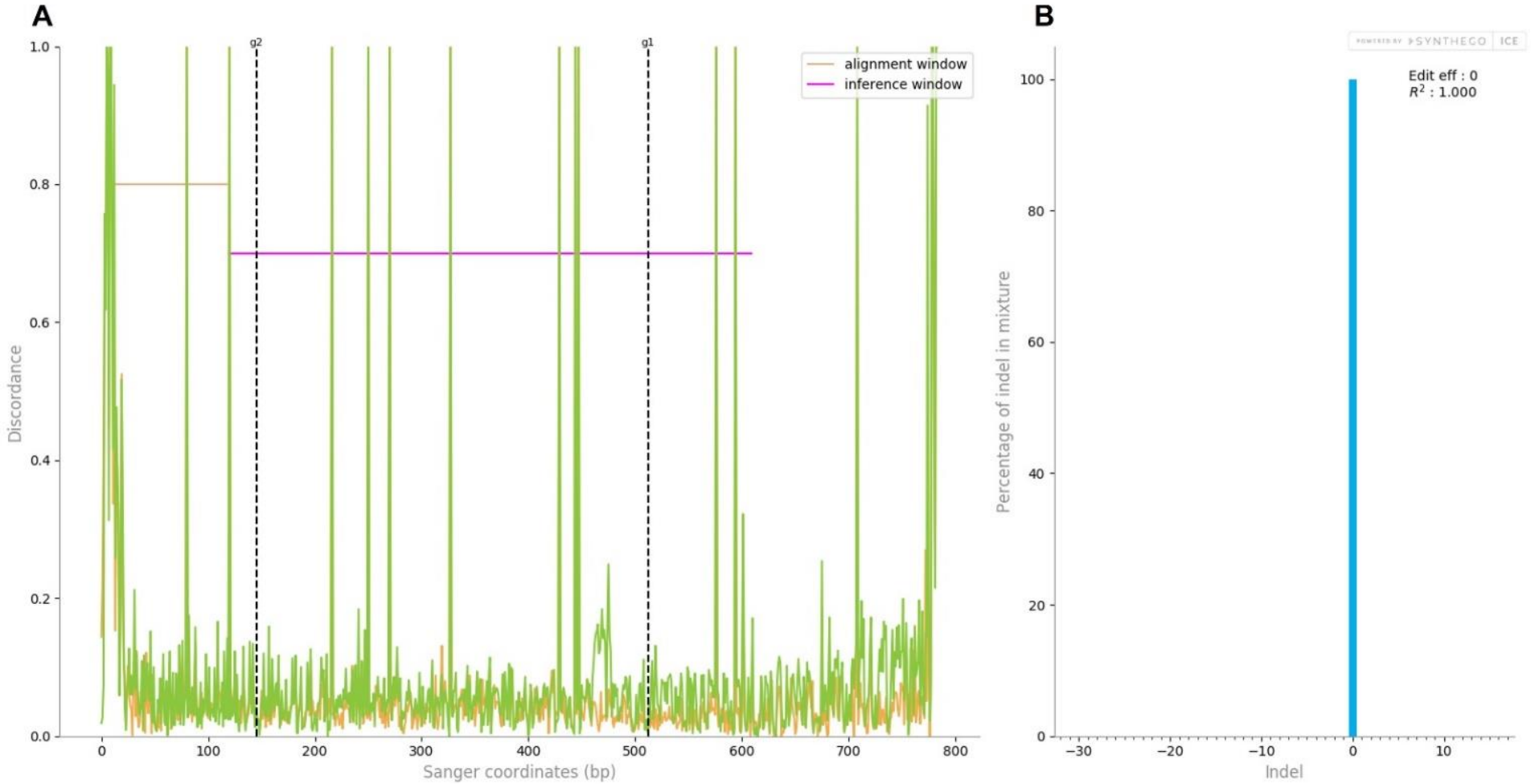
Supplementary Figure 2. Sequences of the region containing the gRNA scaffold and the adjacent sgRNA on the pGEM-sgRNA expression vector. Prior to transcription driven by the T7 promoter, the pGEM-sgRNA expression vector was digested with EcoRI (recognition site underlined in orange). Bases in red squares indicate mismatches. The first row is the sequence of the pGEM-sgRNA vector containing the LCYB-T1 sgRNA; Second row = LCYB-T2; Third row = LCYB-T3. Sanger sequencing performed with the SP6 primer.



Supplementary Figure 3. Plasmid map of the pDIR26H-LCYB expression vector. T-DNA borders are illustrated in red. Kanamycin resistance is conferred by KanR for bacterial selection. Selection for putative transformants is facilitated by bar. ZmUbi promoter drives the expression of the Csy4 and Cas9 endonucleases. PvUbi promoter regulates the multiplex sgRNA expression cassette.



Supplementary Figure 4. Sequence confirmation of the sgRNA cassette of the pDIR26H-LCYB expression vector. 20 bp sgRNA sequences are denoted by blue bars. Each corresponding gRNA scaffold sequence is denoted in the adjacent grey bars.



Supplementary Figure 5. Representative ICE v2 software output for the regenerants transformed with the pDIR26H-LCYB plasmid through Agrobacterium-mediated transformation. A) The measured discordance of the Sanger sequence results the sample vs the control. Vertical dashed lines represent the location of the cut sites of LCYB-T1 and LCYB-T2. B) The indel distribution estimated from the samples. The regenerants were determined to be unedited. Output provided by ICE v2 Software.

Supplementary Figure 6. Sequence confirmation of the uidA *gene (BarR) in a callus that stained blue when subjected to a GUS assay 30 days after being bombarded with the pAHC25.*

Supplementary Table 1. Primers used in various experiments for screening.

Primer Name	Sequence (5'-3')	Amplicon length (bp)	Function
TaCas9-F	tgaagcaactcaagcgccggag	650	Screen for T-DNA insert for pDIR26H-LCBY
TaCas9-R	gcggttcttgtccgacctggtg		
GuspB204-F	catgaagatgcggacttgcg	647	Screen for T-DNA insert for pBRACT204
GuspB204-R	gctaacgtatccacgccgta		
Bar-F	gtctgcaccatcgtcaacc	444	Screen for Bar insert for pAHC25 plasmid
Bar-R	gaagtccagctgccagaaac		
LCYBint-F	tcgtctggcccaacaactac	812	Amplify the region of *TaLCBY* containing all three target sites
LCYBint-R	tccatcgtgcggtttctagac		
LCYB-A-R	ttcgatgtgttggaagcg	1081	Paired with LCYBint-F for amplification of the A homeolog of LCYB
LCYB-B-R	ccatgaagatcttgagatgcag	1202	Paired with LCYBint-F for amplification of the B homeolog of LCYB
LCYB-D-R	atggagcgagcgatacctcg	1361	Paired with LCYBint-F for amplification of the D homeolog of LCYB