Title
Advancing enabling technology and genome editing in monocot crops for disease resistance and sustainability

Author
Poddar, Snigdha

Publication Date
2021

Peer reviewed|Thesis/dissertation

Advancing enabling technology and genome editing in monocot crops for disease resistance and sustainability

By

Snigdha Poddar

A dissertation submitted in partial satisfaction

of the requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jamie H.D. Cate, Co-Chair

Professor Brian J. Staskawicz, Co-Chair

Professor Arash Komeili

Professor David Savage

Fall 2021

Abstract

Advancing enabling technology and genome editing in monocot crops for disease resistance and sustainability

by

Snigdha Poddar

Doctor of Philosophy in Molecular and Cell Biology

University of California

Professor Jamie H.D. Cate, Co-Chair

Professor Brian J. Staskawicz, Co-Chair

It is projected that by the year 2050, global agriculture will need to increase the output of staple crops by 60% to feed the world's rapidly growing population amidst threats posed by climate change and crop disease. One strategy to meet this challenge is to develop new genetic diversity in the germplasm to generate robust and resilient lines with beneficial traits. Conventional breeding has played an essential and major role in crop trait improvement. However, those methods can be difficult, laborious, and time-consuming. The development of effective technology for precision and time-saving plant breeding is required. A breakthrough tool to this end has been CRISPR-Cas9. Noteworthy for its simple sequence-specific programmability, the gene editing system has unleashed remarkable potential for plant biotechnology and functional genomics. Here, we explore several aspects and parameters to advance Cas9-mediated gene editing technology in the essential monocot food crops: rice and wheat. Early-stage plant gene-editing projects benefit greatly from a rapid transient pipeline using protoplasts to evaluate the efficacy of gene editing reagents. We describe here a novel and sustainable method for protoplast isolation from rice tissue and demonstrate their use in ribonucleoprotein (RNP) based Cas9 gene editing assays. Next, we focus on applications of Cas9 technology in wheat to engineer disease resistance. Wheat is a critical target organism because the complexity of its large allohexaploid genome has rendered genetic manipulation by classic methods extremely difficult. Using DNA plasmids encoding CRISPR-Cas9 gene editing reagents, we optimize conditions and demonstrate that all three homoeologous copies of genes in allohexaploid wheat can be simultaneously edited within a single generation. We recognize, however, that DNA plasmid gene editing approaches have a number of limitations including random integration into the plant genome and unpredictability of expression. To address this, we finally present significant improvements to DNA-free Cas9-RNP based gene editing in wheat. We show that increased temperature treatment greatly enhances Cas9-mediated editing efficiency and regenerate transgene-free edited wheat plants at a high rate. Lastly, we utilize this DNA-free gene editing method to generate *de novo* partial resistance to the agronomically important pathogen *Parastagonospora nodorum.*

ACKNOWLEDGMENTS

I am thankful to my advisors, Profs. Brian Staskawicz and Jamie Cate, for their mentorship. Brian's fierce advocacy and knowledge made me feel strongly supported, and Jamie's encouragement and deep passion for science energized me during moments of uncertainty. I also thank Dr. Myeong-Je Cho, who took me under his wing and taught me so much. I will always be grateful to these mentors for the significant roles they have played in my growth as a scientist.

I thank my thesis committee members, Profs. Arash Komeili and Dave Savage for their enthusiasm for my work, their continuous feedback, and for being kind and inspiring scientists.

My dissertation work was only made possible by the many brilliant scientists I had the honor of working with. Foremost, thank you to Jackie Tanaka, whose hard work and dedication is unmatched. To my co-authors, Doug Dahlbeck, Eunyoung Seo, Katherine Running, Justin Faris, Tim Friesen, Giovanna Cruppe, and Barbara Valent, thank you so much for your contributions and help. And to the members of my two labs, past and present, my colleagues, and friends, thank you for being such wonderful, intelligent, and warmhearted people.

Thank you to Steven, whose continuous care and support carried me through every step of this journey. Thank you to my mom, my dad, and my brother, whose love and dedication shaped the person I am today, kept me grounded, and continues to motivate me to be the best version of myself. I am profoundly grateful for my family.

TABLE OF CONTENTS

CHAPTER 1. Introduction ……...1

CHAPTER 2. Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays ……..…..3

2.1 Introduction……3
2.2 A sustainable protoplast isolation method with optional pause points……..…4
2.3 Protoplast viability over time……8
2.4 Transfection efficiency over time…….....9
2.5 Gene editing via Cas9 ribonucleoprotein transfection……11
2.6 Discussion……11
2.7 Materials and Methods……12

CHAPTER 3. Cas9-mediated homozygous knockouts of rice blast susceptibility gene orthologs in wheat……14

3.1 Introduction……..14
3.2 Identification of *Pi21* and *Bsrk-1* wheat orthologs ….……....15
3.3 Generating mutations in *TaPi21* ……..……....18
3.4 Generating mutations in *TaBsrk-1* ……...19
3.5 Analysis of editing outcomes and temperature treatment in *Bsrk-1* targeted T_0 regenerants ……20
3.6 Knockout of *Pi21* or *Bsrk-1* wheat orthologs do not confer resistance to *Xanthomonas translucens* or *Magnaporthe oryzae pathotype Triticum*……21
3.7 Discussion……23
3.8 Materials and Methods……24

CHAPTER 4. Impact of temperature and time on DNA-free Cas9-ribonucleoprotein mediated gene editing in wheat protoplasts and immature embryos ……27

4.1 Introduction…….....27
4.2 Cas9-RNP transfection and the effect of temperature in wheat protoplast gene editing ……28
4.3 Biolistic Cas9-RNP delivery and the effect of temperature in wheat immature embryo gene editing……29
4.4 Cas9-RNP mediated editing is sustained over time……32
4.5 Relative editing rates in protoplasts correlate linearly with editing rates in M_0 regenerants from bombarded immature embryos ……..33
4.6 Cas9-RNP mediated knockout of *Parastagonospora nodorum* necrotrophic effector sensitivity genes……34
4.7 Discussion……36
4.8 Materials and Methods……39

CHAPTER 5. Concluding remarks……43

REFERENCES……44

CHAPTER 1. Introduction

To feed a rapidly growing human population, mitigate the effects of climate change, and engineer resistance to plant pathogens, new approaches are needed to increase the speed and precision of crop varietal development. Over the course of nearly 10,000 years of agriculture, plant breeding has taken advantage of natural genetic diversity existing in crops through the development of traditional strategies, such as marker-assisted selective propagation and breeding to select for desirable traits[1,2]. These strategies, however, are limited by tremendous inputs of time, resources, labor, and issues of genetic linkage drag [3]. There exists demand for modern techniques by which endogenous genes can be edited with high efficiency and sequence specificity without the need for extensive crossing and backcrossing to achieve the desired genotype [4]. CRISPR-Cas9, an RNA-guided endonuclease [5], has revolutionized the field of plant genome editing due to its efficient sequence specific DNA double stranded cleavage activity [6]. The strategy takes advantage of an organism's DNA repair mechanisms upon double stranded breaks [7] to direct editing in the form of error-prone non-homologous end joining (NHEJ) produced insertions or deletions. Applications of this technology to food crops can greatly accelerate production of allelic variation, diversity, and improvements to agriculture.

The dissertation work detailed here entails investigations of the CRISPR-Cas9 genome editing landscape in monocot plant cells and advancements to enabling technology and genome editing processes in monocot crops. The studies are motivated by efforts to engineer disease resistance and increase sustainability in agriculture.

One such piece of enabling technology is an efficient protoplast isolation and transfection pipeline. Protoplasts, which are plant cells that have been enzymatically treated to remove cell walls, are important tools to study gene expression, metabolic pathways, and gene editing parameters in plants. Although rice protoplasts can be isolated from germinated seedlings or cell suspension culture, preparation of those donor tissues can be inefficient, time-consuming, and laborious [8-12]. Additionally, the lengthy process of protoplast isolation and transfection needs to be completed in a single day. In Chapter 2, we report a protocol for the isolation of protoplasts directly from rice calli, without using seedlings or suspension culture. The method was developed to employ discretionary pause points during protoplast isolation and before transfection. The protoplasts produced by this method are competent for transfection of both plasmids and ribonucleoproteins (RNPs). Cas9 RNPs were used to demonstrate the utility of these protoplasts to assay genome editing *in vivo*. We describe a highly effective and accessible method to isolate protoplasts from callus tissue induced from rice seeds. The method utilizes donor materials that are resource-efficient and easy to propagate, permits convenience via pause points, and allows for flexible transfection days after protoplast isolation. It provides an advantageous and useful platform for a variety of *in vivo* transient transfection studies in rice including Cas9 gene editing assays.

The study described in Chapter 3 focuses on editing genetically complex allohexaploid wheat using particle bombardment of plasmids encoding Cas9. In addition to tackling gene editing in the complicated wheat genome, we were driven to explore a potential strategy to combat wheat blast disease. Blast disease, caused by the fungus *Magnaporthe oryzae*, is a destructive disease that leads to substantial loss of annual rice and wheat harvests [13]. Critically, wheat blast emerged in South America in 1985 and has since spread to Asia, threatening annual crop harvests [14]. We deployed gene editing in wheat to simultaneously knock out two sets of three homoelogous wheat genes with orthology to rice genes characterized to control resistance to blast disease. Loss of

function of these negative regulators of resistance leads to broad spectrum resistance in rice that is more durable than that conferred by effector specific resistance genes [15,16]. Given the family level phylogenetic relationship between rice and wheat, we reasoned that the wheat orthologs we identified could retain similar function to the rice genes. Ultimately, though, we found that complete knockout of these wheat genes did not confer resistance to disease in wheat. However, by optimizing Cas9 guide RNAs and developing high frequency transformation by plasmid microparticle bombardment and heat treatment, we generated homozygous susceptibility gene knockouts across all three genomes of a wheat plant in the first generation and quantified the incidence rate of generating a full spectrum of genotypes from nearly 100 transformed plants.

While *Agrobacterium* or particle bombardment-based delivery of plasmids encoding Cas9 and guide RNA (gRNA) is common, it requires optimization of expression and often results in random integration of plasmid DNA into the plant genome [17-21]. Recent advances have described gene editing by the delivery of Cas9 and gRNA as pre-assembled RNPs into various plant tissues, but with moderate efficiency in resulting regenerated plants [22-24]. In Chapter 4, we describe major improvements to Cas9-RNP mediated gene editing in wheat. We demonstrated that Cas9-RNP assays in wheat protoplasts are a fast and effective tool for rational selection of optimal gRNAs for gene editing in regenerable immature embryos (IEs), and that high temperature treatment enhances gene editing rates in both tissue types. We also showed that Cas9-mediated editing persisted for at least 14 days in gold particle bombarded wheat IEs, contrary to previous hypotheses that the protein is degraded *in vivo* within 72 hours [25]. We regenerated edited wheat plants in this work at high rates in the absence of exogenous DNA and selection. With this method, we produced knockouts of a set of three homoeologous genes as well as two pathogenic effector susceptibility genes that resulted in insensitivity to corresponding necrotrophic effectors produced by the pathogen *Parastagonospora nodorum*. The establishment of highly efficient, DNA-free gene editing technology holds promise for accelerated trait diversity production in an expansive array of crops.

With this work, we explored numerous aspects and parameters of Cas9-mediated gene editing in the essential food crops: rice and wheat. We developed new methods for protoplast isolation and establish their use in gene editing assays as an inexpensive, fast, and accurate method to predict gene editing efficiency in regenerable tissue. We unveiled the importance of heat treatment not only with plasmid-based, but also RNP-based gene editing using Cas9, and showed that all three homoeologs of a gene in allohexaploid wheat can be successfully targeted for mutation within the first generation after transformation of IEs. Finally, we look to the future of crop gene editing by greatly improving upon selection and DNA-free, Cas9-RNP mediated gene editing of wheat, setting the stage for utilization of this technology in vegetatively propagated crops.

CHAPTER 2. Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays

2.1 Introduction

Rice (*Oryza sativa* L.) is a vital crop that provides staple calories for approximately half of the global population and is a model organism for basic research of monocotyledon plant biology [26,27]. Amidst rapid population growth, climate change, and threats posed by pests and pathogens, the need to address food security via improved agricultural output is high. To meet these challenges, it is important to advance basic scientific understanding of plant processes, molecular machinery, and genetics. Concurrently, those advances can be applied and developed via biotechnological efforts to improve plants for increased yield, new genetic diversity, insect resistance, disease resistance, drought tolerance, herbicide tolerance and other agronomically important traits [28].

Much of this work, particularly early-stage experiments, can be hastened via robust protoplast systems. The delivery of DNA or RNPs into plant tissue for biological assays is impeded by the presence of a rigid cell wall surrounding each cell. Enzymatic digestion of the cell walls followed by a purification process yields membrane-bound protoplasts [29]. These cells are useful and versatile gene expression systems competent for transfection of exogenous genetic material. Other experimental platforms exist in plants, such as heterologous expression [30,31] and stable and transient transformation by *Agrobacteria* [32,33] or particle bombardment [34,35]. However, heterologous expression systems can be linked to a caveat of aberrant characteristics [36], and stable transformation requires significant resources and can be superfluous for some applications including, but not limited to, studies in protein subcellular location and protein-protein interactions.

Protoplast studies are uniquely suited for facile, rapid, and high throughput *in vivo* assays to examine gene expression as well as to evaluate genome editing efficacy. The advent of targeted plant genome editing, mediated by various sequence-specific nucleases, is a powerful biotechnological development that has hastened plant gene function studies and crop development [37]. The CRISPR/Cas9 system, in particular, has provided significant utility due to its simplicity and versatility [5,38]. A critical factor driving editing efficiency is the guide RNA (gRNA) sequence that guides specific Cas9 cleavage of genomic DNA. Because generating stable genome-edited plants is complex and labor intensive, it is beneficial to first determine the most effective gRNAs *in vivo* as well as identify the range of mutations made through a simple and rapid protoplast pipeline.

Generally, protoplasts are isolated from leaves or germinated seedlings for transient transfection in several plant species [39-43]. Rice protoplasts can be isolated from cell suspension culture [8,9] as well as seedlings [10-12]. While effective, these methods can be time-consuming and laborious. Isolation from seedlings requires 80-120 fresh seedlings per protoplast preparation, which can deplete seed pools quickly. Meticulous manual slicing of the plant material into small strips is also a critical step in the protocol. The blade must be changed regularly to ensure clean cuts, as any bruising of the leaf tissue leads to a lower yield of healthy protoplasts. Meanwhile, the establishment and maintenance of cell suspension culture requires experienced skill to select proper callus morphologies and are vulnerable to contamination [8,44,45]. Furthermore, transfections are performed immediately after isolation, raising an additional component of time sensitivity.

In the present study, we describe a highly efficient method to isolate rice protoplasts from callus tissue derived from dry seeds. The induction and proliferation of calli is straightforward, sustainable, and sterile. We analyze protoplast viability and transfection competence over time,

utilize the method for a genome editing assay, and demonstrate that this method provides convenience via pause points during protoplast isolation and is permissive for transfection of protoplasts for multiple days after initial cell wall digestion of calli.

2.2 A sustainable protoplast isolation method with optional pause points

Existing methods for protoplast isolation from rice using germinated seedlings and suspension cultures are valuable and well described [8-10,44-47]. However, the protocol using germinated seedlings consumes seeds at a high rate and the suspension culture protocol requires the technical and labor-intensive know-how of maintaining a suspension culture. In contrast, the present protoplast isolation protocol using rice calli described here is highly effective and accessible because callus induction from mature seeds is straightforward and fast. We could generate about 5 g of calli 1 month after callus induction from 30 mature seeds. These tissues can be proliferated to about 30-40 g of calli after another month, and 150 to 200 g of calli are available 3 months after the initial callus induction. These tissues can be further proliferated with additional rounds of transfers and can be utilized at any stage on a large scale. Furthermore, the methods require an uninterrupted and lengthy workflow from donor tissue digestion through transfection performed on the isolated protoplasts.

Here, techniques are outlined for a branched method with built-in optional pause points that allow for consistent and efficient procurement of healthy protoplasts that may be used gradually, over the course of several days, for downstream transient assays. The donor tissue for the isolation of protoplasts are calli induced from seeds and regularly sub-cultured on solid OsCIM2 callus induction media (Table 1). Calli were also induced from immature embryos in this manner, with comparable outcomes. In general, callus tissue propagated for more than six months could be used in this method. As a result, the donor tissue becomes available at an exponential rate once initiated, abrogating the obstacle of donor material availability for this procedure.

Table 1: Composition of medium/solutions used for protoplast isolation and transfection

Medium/Solution Name	Compositions
OsCIM2	3.99 g/L Chu's N6 Basal Medium with Vitamins (C167, PhytoTechnology Laboratories, Lenexa, KS, USA), 30 g/L maltose, 0.1 g/L myo-inositol, 5 uM $CuSO_4$, 0.3 g/L casein enzymatic hydrolysate, 2.5 mg/L 2,4-D, 0.2 mg/L BAP, 0.5 g/L L-Proline, 0.5 g/L L-Glutamine, pH 5.8, solidified with 3.5 g/L Phytagel (P8169; Sigma-Aldrich Corp., St Louis, MO, USA). Autoclaved.
Digestion solution	10 mM MES pH 5.7, 0.6 M mannitol, 1.5% cellulase Onozuka R-10 (Yakult, Tokyo, Japan), 0.1% pectolyase (or 0.75% macerozyme R-10) (Yakult, Tokyo, Japan), 10 mM $CaCl_2$, 4 mM 2-mercaptoethanol, 0.1% bovine serum albumin. *Special instructions:* MES, mannitol, H_2O, cellulase R10, and pectolyase were stirred and incubated at 55°C for 10 minutes. The solution was cooled to room temperature, and $CaCl_2$, 2-mercaptoethanol, and bovine serum albumin were added in and gently mixed.
W5 solution	2 mM MES pH 5.7, 154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$
MMG solution	4 mM MES pH 5.7, 0.6 M mannitol, 15 mM $MgCl_2$
WI solution	4 mM MES pH 5.7, 0.4 M mannitol, 4 mM KCl
PEG-$CaCl_2$ solution	0.4 M mannitol, 100 mM $CaCl_2$, 40% (wt/vol) PEG4000 (81240; Sigma-Aldrich Corp., St Louis, MO, USA)

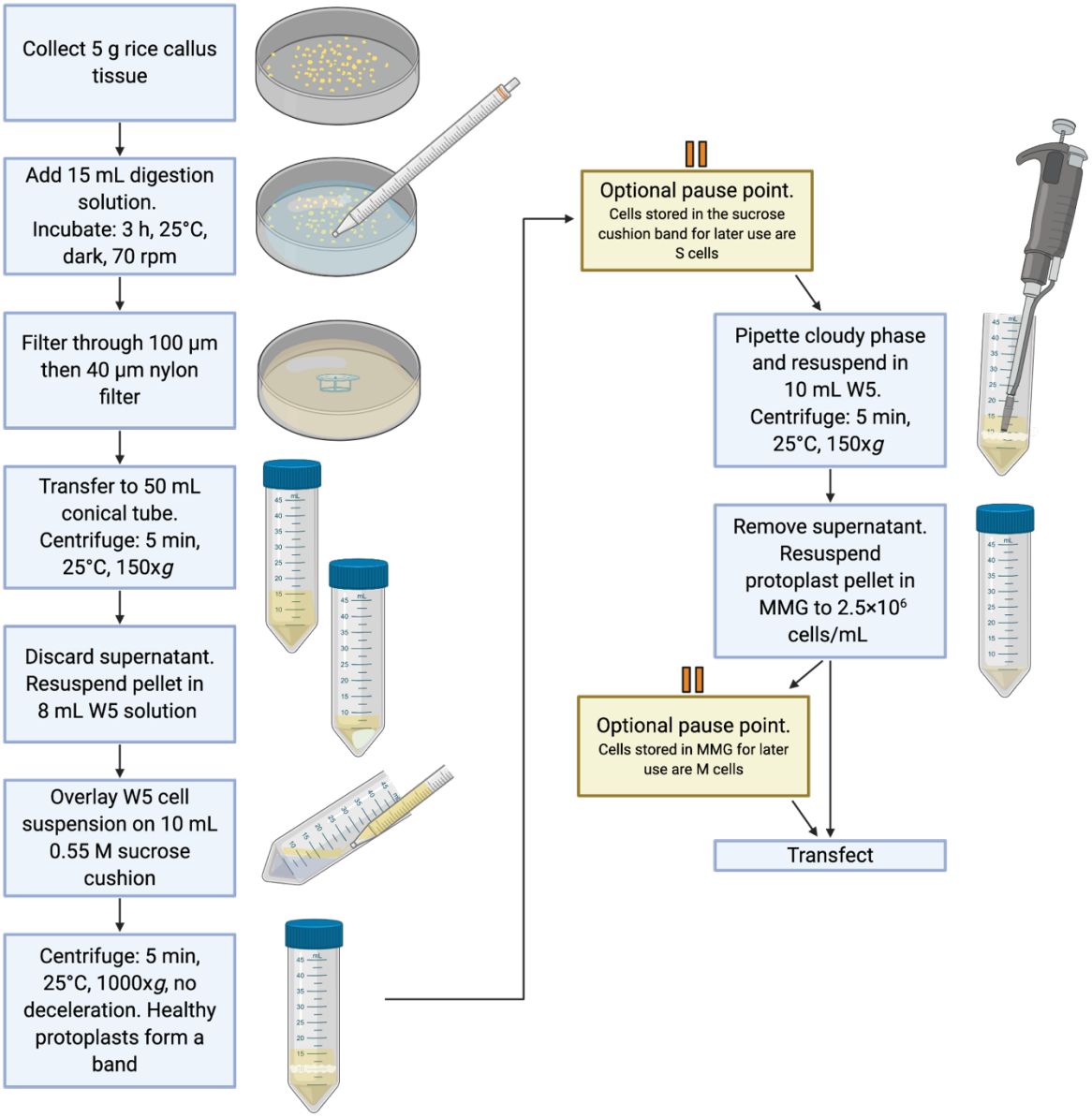


Figure 1. Graphical depiction of the protoplast isolation workflow

The workflow for the protoplast isolation protocol produced from this study is portrayed in Fig. 1. We gathered 5 g of compact pale-yellow rice callus tissue (Fig. 2A) and used a scalpel or metal spatula to bring all the pieces to roughly the same size. Careful slicing and razor exchanges were not needed. An enzymatic cocktail of 1.5% cellulase R10 and 0.1% pectolyase or 0.75% macerozyme R10 resulted in a successful breakdown of rice callus tissue cell walls while maintaining healthy viable protoplasts (Figs. 2B, C). An additional step of vacuum infiltration of the digestion solution with the donor tissue, an approach utilized in other protocols [11,48], was unnecessary for our method, eliminating a common step, decreasing equipment load, and increasing simplicity. Rather, we could simply incubate the callus tissue with 15 mL digestion solution with gentle shaking at 70 rpm for 3 hours, less time than is required for seedling-derived cells. After digestion, protoplasts were isolated from spent tissue via filtration

(Fig. 2D) and centrifugation through a 0.55 M sucrose cushion. A gentle overlay of the cell suspension onto the sucrose was found to be a crucial step for optimal yield. If the cells were handled crudely and dropped with a force that significantly broke the surface tension of the sucrose, the ultimate protoplast yield could be diminished. After centrifugation of the cell suspension through 0.55 M sucrose, healthy protoplasts separated from debris and accumulated to form a dense band of purified protoplasts at the W5 - sucrose interface (Fig. 2E), bringing the protocol to its first optional pause point.

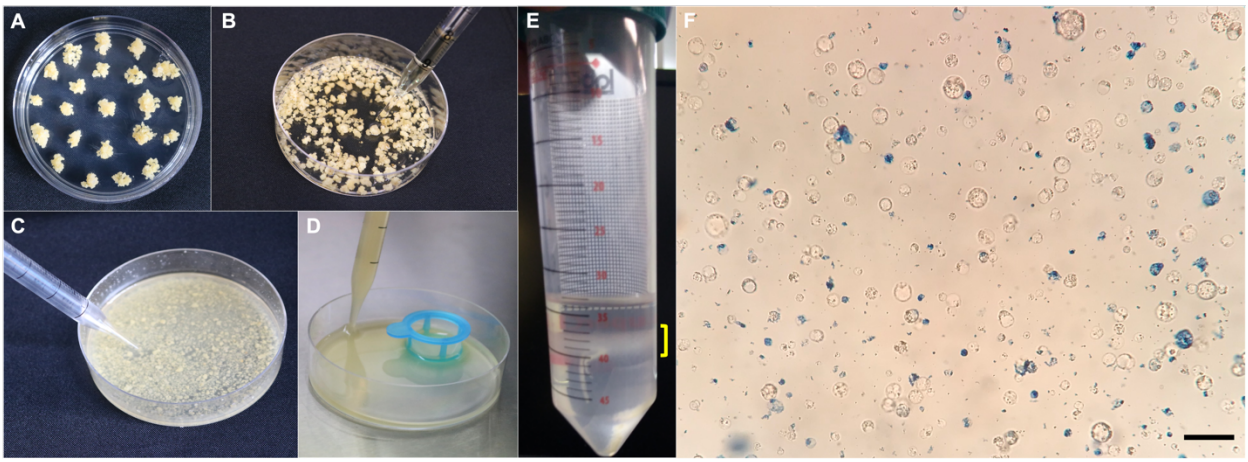


Figure 2. Isolation of protoplasts from rice calli induced from mature seeds. (A) Donor tissue for protoplast isolation were translucent, pale yellow, and nodular calli propagated on OsCIM2 medium. (B) To degrade the tissue cell walls, digestion solution was added. (C) A visual indication of successful enzymatic digestion was a milky appearance of the solution after three hours of gentle shaking. (D) Large particles and spent tissue were removed from the protoplast solution via cell strainer filtration. (E) Protoplasts formed a visible band, marked by a yellow bracket, after centrifugation through a 0.55 M sucrose cushion. (F) Protoplasts derived from rice calli. Healthy cells are round and colorless. Dead cells and debris are stained by Evans Blue. Bar = 50 µm

Here, the method could be paused for one or more days. The band of protoplasts could be left undisturbed at the interface for processing at a later time or handled immediately. The protoplasts produced from the utilization of this pause point are termed “S protoplasts.”

To isolate protoplasts from the sucrose cushion, the entirety of the cloudy phase band containing the protoplasts was gently pipetted out. This was followed by final washing and centrifugation steps to maximize the purity of the protoplasts and eliminate cellular debris. Finally, protoplasts were resuspended in MMG solution to a concentration of 2.5 x 10^6 protoplasts/mL. Protoplasts could be transfected immediately, or the second optional pause point could be employed—storing the protoplasts in MMG solution, termed “M protoplasts,” until transfection at a later time.

Protoplasts isolated from the band on the same day as digestion of the donor tissue cell walls were referred to as “Sucrose Cushion Day 0/ MMG Day 0” (S/M0) protoplasts. Those isolated from the interface one, two, three, or 7 days after digestion were designated S1, S2, S3, or S7 protoplasts, respectively. S/M0 protoplasts, stored in MMG solution and utilized in experiments over the following one, two, three, or 7 days after digestion were labeled “MMG Day 1” (M1), M2, M3, or M7 protoplasts.

2.3 Protoplast viability over time

To ensure the utility of this branched method for protoplast isolation, Evans blue staining was used to quantify viable protoplasts in all isolations from the sucrose cushion (S protoplasts) as well as protoplasts stored in MMG solution over time (M protoplasts) (Fig. 2F). Healthy intact protoplasts derived from this method are colorless, spherical, and resistant to staining. The viability assay indicated that S/M0 isolates yielded the greatest number of live protoplasts, with a gradual decrease with the increasing age of the protoplast-containing sucrose cushion. S/M0 isolates contained approximately 2.5 times the number of protoplasts as S7 isolates. However, it is notable that the order of magnitude for the protoplast count remained unchanged between S/M0 and S7. We show that from 5 grams of rice callus donor tissue, this method yields, on average, 9.8×10^6 live protoplasts if isolated on day 0 (S/M0) and 3.9×10^6 live protoplasts if isolated on day 7 (S7) (Fig. 3A). This translates to approximately 20 transfection reactions with S/M0 protoplasts, and 8 transfection reactions with S7 protoplasts. To compare, 100-120 finely sliced rice seedlings are required to obtain approximately the same number of protoplasts as an S/M0 isolation using 5 g of calli by the current method [46], and which cannot be stored for future use.

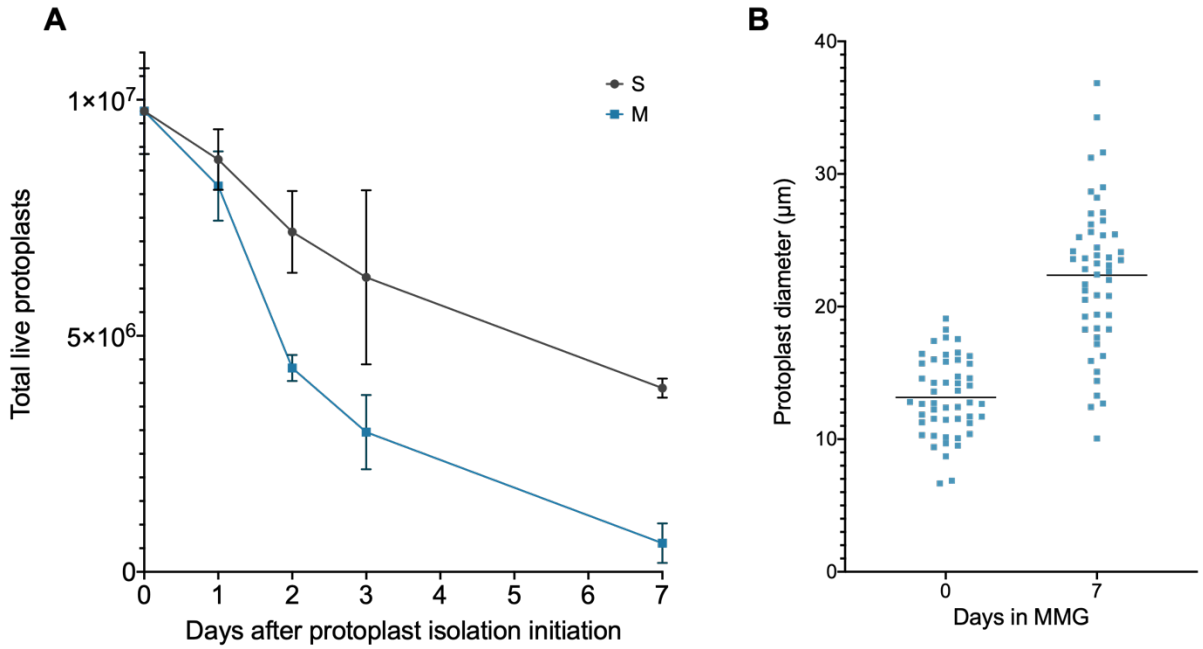


Figure 3. Protoplast viability and size over time. (A) Viability of protoplasts was measured by counting protoplasts unstained by Evans Blue dye on a hemocytometer. Total live protoplasts were calculated by first determining protoplast density, then multiplying by the total volume of protoplasts from the isolation. Counts were performed in triplicate. The means are plotted, and error bars indicate standard deviation. (B) A random sampling of 50 protoplasts were measure for diameter at 0 and 7 days in MMG. Each measurement was plotted individually, and the means were indicated by a horizontal line.

Viability of M protoplasts was also tracked over time. Though viability decreases appreciably from day 2, the concentration of viable M1 protoplasts is comparable to S1 protoplasts and only mildly reduced from S/M0 protoplasts, making them an acceptable option for use in assays (Fig. 3A). It was also noted that protoplasts held in MMG solution for 7 days appeared approximately 1.7X larger (Fig. 3B). This may be attributed to cell growth or osmotic swelling.

However, the larger protoplasts displayed a characteristically healthy spherical shape, unstained by Evans blue, suggesting that osmotic stress was not occurring.

2.4 Transfection efficiency over time

Both quantity and quality of protoplasts are critical factors for downstream experiments. In existing methods, transfection is performed only on freshly isolated protoplasts. Here, the transfection efficiency of both S and M protoplasts of different ages were assayed via PEG-mediated transfection of pAct1IsGFP-1, a GFP overexpression plasmid [49].

First, S and M transfection pools were imaged for GFP expression 1 and 2 days after transfection (Fig. 4). Protoplasts aggregate over time and the 1 mL pools were not pipetted for homogeneity. Rather, a fluorescence stereomicroscope was used to manually scan the sample and gather representative images in areas with moderate density of protoplasts. Strong GFP fluorescence was detected in both S and M cells 1 and 2 days after transfection.

Transfection efficiency was calculated 2 days after plasmid transfection as a percentage of GFP positive protoplasts over total live protoplasts, as determined by fluorescence microscopy and Evans Blue staining on a hemocytometer (Fig. 5). For S/M0 protoplasts, transfection was highly efficient, producing 73.5% GFP expressing protoplasts. S1 transfection efficiency was comparable, at 69.5%. Taken together with the results from the previously described viability assay, the data suggest that S1 protoplasts are comparable in value to freshly isolated S/M0 protoplasts. This finding facilitates novel flexibility in research, allowing assays to be performed 24 hours after initiation of the protoplast isolation method with little to no loss of efficacy and data. Though transfection efficiency declines over time for both S and M protoplasts, it does not fall below 15% within 7 days (Fig. 5). Moreover, it is conceivable that certain assays, for example protein localization, do not require optimal transfection efficiency or viability. The data provided here allow for the informed design and versatile scheduling of protoplast experiments with a quantified summary of expected losses of viability and transfection efficiency over time.

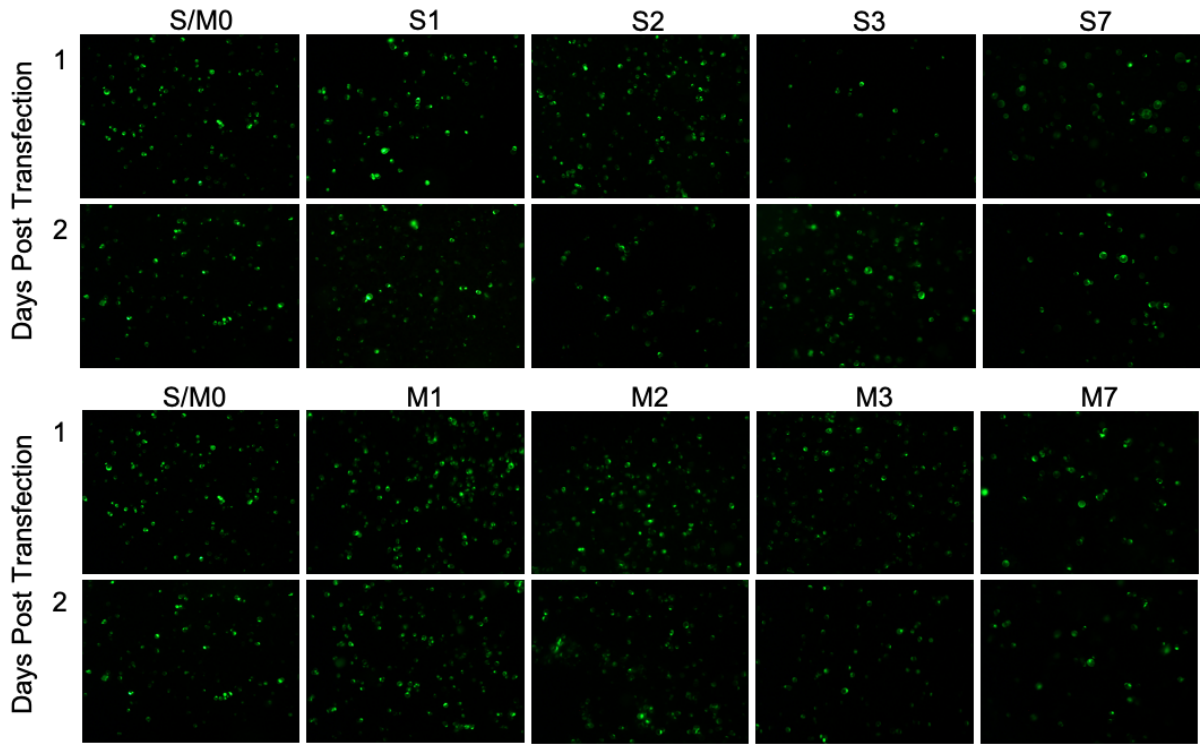


Figure 4. GFP expression in S and M callus-derived protoplasts of different ages. S (top panel) and M (bottom panel) protoplasts of different ages were transfected with pAct1sGFP-1 and imaged for GFP fluorescence. Images were taken at 80X magnification on a Leica M165 fluorescence microscope. Transfected protoplasts were in 1 mL WI solution pools in 12-well culture plates.

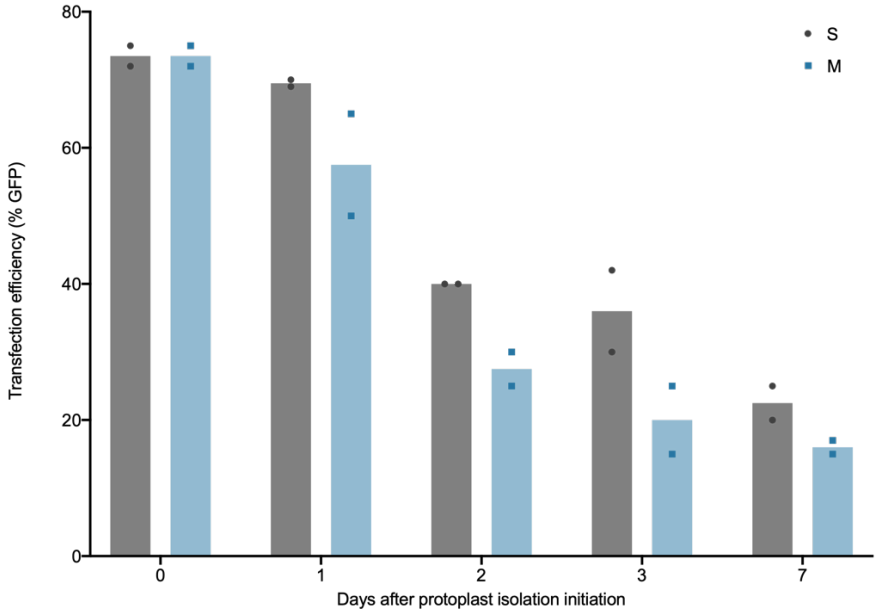


Figure 5. Transfection efficiency in S and M protoplasts isolated from rice calli. The percentage of GFP fluorescence-positive protoplasts were calculated after transfection with pAct1IsGFP-1 to determine plasmid DNA transfection efficiency in S and M callus-derived rice protoplasts of different ages. The transfections were performed in duplicate, with each data point shown. The bar indicates the mean.

2.5 Gene editing via Cas9 ribonucleoprotein transfection

Given the flourishing field of genome editing, it was critical to ensure that this method was suitable for such studies. To demonstrate this, S/M0 as well as S1 and M1 protoplasts were transfected with *in vitro* assembled Cas9-gRNA ribonucleoproteins targeting a single locus (Fig. 6). For comparison with protoplasts isolated via a previously published method [11], protoplasts derived from rice seedlings were also transfected. Editing at the Cas9 cleavage site was identified and quantified through NGS. Editing rates for S/M0 protoplasts and seedling-derived protoplasts were similar (Fig. 6), indicating that the present protocol can be used confidently in genome editing studies.

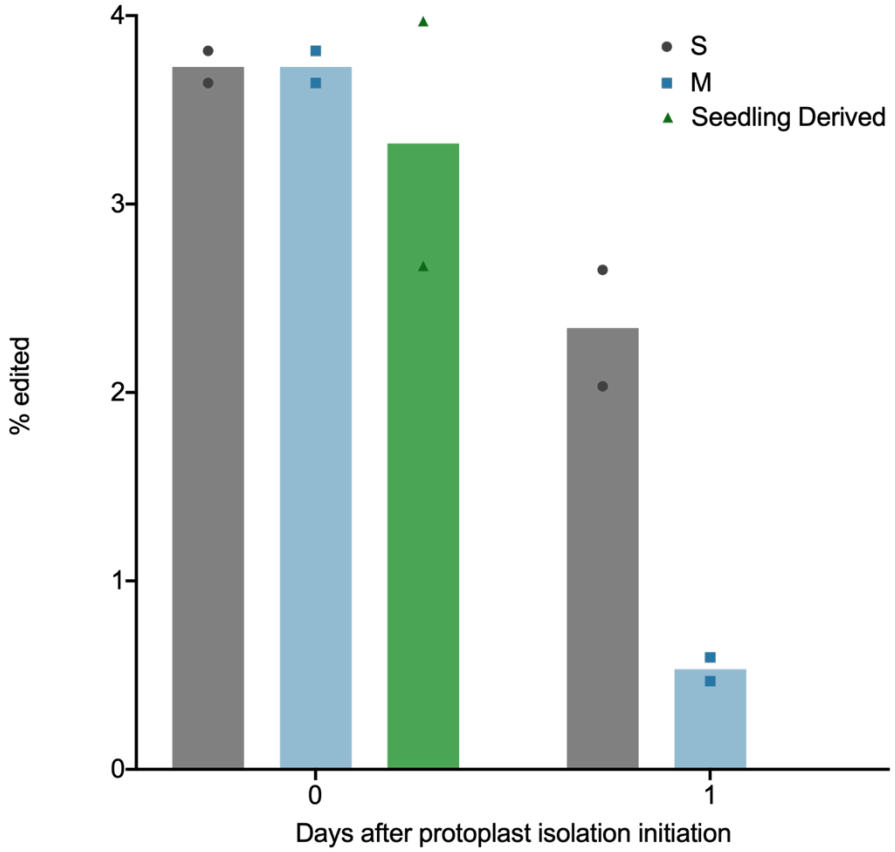


Figure 6. Genome editing efficiency in rice protoplasts isolated from rice calli. Editing efficiency of Cas9 and gRNA ribonucleoproteins in S and M callus-derived rice protoplasts of different ages was compared to seedling-derived rice protoplasts**.**

2.6 Discussion

The current study describes an embryogenic rice callus-derived protoplast isolation method that avoids the growth of numerous rice seedlings or induction and maintenance of a suspension culture. It also includes optional pause points during and after protoplast isolation. The ability to pause the protocol as well as utilize viable stored M protoplasts increases flexibility in schedules and experimentation for researchers. Because the process of obtaining donor material through

isolation of protoplasts and transfection is performed under sterile conditions in its entirety, the protoplasts can be maintained without contamination for time course experiments from transfection through subsequent 7 days or longer. In addition, we demonstrate that the protoplasts produced from this method are competent for transfection of both DNA and RNPs, suitable as transient expression systems, and effective for CRISPR-Cas9 based genome editing assays.

2.7 Materials and Methods

Plant materials

Plants of rice (*Oryza sativa* L.) cultivar, Nipponbare, were grown in a greenhouse at 16/8-h photoperiod intervals (250-300 µmol $m^{-2}s^{-1}$), 27 °C and 22 °C, respectively.

Reagents and solutions

Recipes for callus induction media, digestion solution, W5 solution, MMG solution, WI solution, and PEG-$CaCl_2$ solution are listed in Table 1. All solutions are 0.2 µm filter sterilized.

Callus induction and subculture

Mature seeds of rice were used to induce callus tissue. Briefly, dehulled mature seeds were surface sterilized for 15-20 minutes in 20% (v/v) bleach (5.25% sodium hypochlorite) plus one drop of Tween 20 followed by three washes in sterile water and placed on OsCIM2 callus induction medium (Table 1). After 7-14 days, the coleoptiles and endosperm tissues were removed from the mature seeds and the translucent, pale-yellow nodular calli were transferred onto fresh OsCIM2 every three to four weeks for subculture.

Protoplast isolation

All exposed steps were performed under sterile conditions within a laminar flow hood. After enzymatic digestion of tissue, all pipetting of protoplasts was performed with sterile 1 mL tips with the top 0.25 cm removed. Five to six grams of compact, nodular callus tissue were collected from subcultured OsCIM2 plates and gently crumbled using the edge of a metal spatula or scalpel in a deep 25×100 mm petri dish with 15 mL of digestion solution. The petri dish was incubated in the dark in a room temperature shaker at 70 rpm for 3 hours until the digestion solution appeared milky.

The protoplast-filled digestion solution was first filtered through a Falcon 100 µm nylon cell strainer (352360; BD Biosciences, San Jose, CA, USA) in a sterile petri dish and then through a Falcon 40 µm nylon cell strainer (352340; BD Biosciences). The protoplast solution was transferred to a 50 mL conical tube and centrifuged for 5 minutes at room temperature at 150x*g*. The supernatant was discarded, and the protoplast pellet was gently resuspended in 8 mL W5 solution. Separately a fresh 50 mL conical tube with 10 mL of 0.55 M sucrose was prepared. The cell suspension was gently pipetted onto the sucrose cushion such that the cell suspension floated on top, then centrifuged at 1000x*g* without deceleration for 5 minutes.

At this stage, the isolation process could be paused until subsequent days, or continued immediately. If the pause point was utilized, the tube was stored at room temperature, upright, undisturbed, and away from direct light.

The intermediate cloudy phase, containing live protoplasts, was pipette extracted and mixed with 10 mL W5 solution in fresh tubes. The suspension was centrifuged for 5 minutes at room temperature at 150x*g*. The supernatant was removed, and the pellet gently resuspended in 5

mL of MMG solution. The suspension was once again centrifuged for 5 minutes at room temperature at 150x*g*. The protoplast pellet was resuspended in 4 mL of MMG, or enough to bring the final cell concentration to 2.5×10^6 cells/mL as calculated by microscopy on a hemocytometer.

Protoplast viability

One µL of 1% Evans blue (E2129; Sigma-Aldrich Corp., St Louis, MO, USA) was added to 25 µL protoplast suspension. The protoplasts were viewed on a hemocytometer under a light microscope. Live protoplasts, which remained unstained, were counted and total live protoplasts per milliliter were calculated. Dead protoplasts and debris were stained blue.

Protoplast transfection

PEG-mediated transfection was performed, guided by previously published methods [11,50] with modifications. In a sterile 1.5 mL tube, 10 µg of 250 ng/µL plasmid DNA pAct1IsGFP [49] were added to 200 µL of protoplasts suspension (5×10^5 total cells), gently flicked and inverted to mix thoroughly, and incubated at room temperature in the dark for 5 minutes. Two hundred forty µL of PEG-$CaCl_2$ solution were added, and the tube inverted gently several times until fully mixed. This was further incubated at room temperature in the dark for 20 minutes. After incubation, 800 µL of W5 solution were added to stop the reaction, inverted gently several times until fully mixed, and centrifuged at 200x*g* for 5 minutes. The supernatant was carefully pipetted for removal, reserving the protoplast pellet. The protoplast pellet was resuspended with gentle inversions and minimal pipetting in 1 mL WI solution and transferred into a 12-well tissue culture plate. The plate edges were sealed with parafilm and incubated in the dark at 26 °C for 48 hours until they were utilized for light microscopy to measure protoplast viability on a hemocytometer and GFP fluorescence using a Zeiss Axio Imager (Carl Zeiss Microscopy LLC, White Plains, NY) and a Leica M165 fluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL).

Rice protoplast genome editing and amplicon next generation sequencing analysis

Protoplasts were transfected with Cas9 RNPs based on a previous study, with modifications [51]. A 1:1 ratio of tracrRNA and target specific crRNA (Integrated DNA Technologies, Coralville, IA) were annealed to form gRNA. Ten µg Cas9 protein (Macrolab, University of California, Berkeley, CA) and 10 ug gRNA were incubated at 37 °C for 20 minutes in a total 25 µL to assemble the Cas9 RNPs. Protoplast transfection was performed, as described above, using 25 µL RNPs instead of plasmid DNA. Forty-eight hours post-transfection, the protoplasts were harvested for CTAB-chloroform genomic DNA extraction. To determine mutation rates by amplicon sequencing, PCR was performed with target specific primers, amplifying approximately 300 bp around the cut site using Q5 High-Fidelity (New England Biolabs, Ipswich, MA) polymerase. Primers contained a 5'-stub compatible with Illumina NGS library preparation. PCR products were ligated to Illumina TruSeq adaptors and purified. Libraries were prepared using a NEBNext kit (Illumina) according to the manufacturer's guidelines. Samples were deep sequenced on an Illumina MiSeq at 300 bp paired-end reads to a depth of approximately 10,000 reads per sample. Cortado (https://github.com/staciawyman/cortado) was used to analyze editing outcomes. Briefly, reads were adapter trimmed and then merged using overlap to single reads. These joined reads were then aligned to the target reference sequence. Editing rates are calculated by counting any reads with an insertion or deletion overlapping the cut site or occurring within a 3 base pair window on either side of the cut site. SNPs occurring within the window around the cut site are not counted. Total edited reads are then divided by the total number of aligned reads to get percent edited.

CHAPTER 3. Cas9-mediated homozygous knockouts of rice blast susceptibility gene orthologs in wheat

3.1 Introduction

Wheat (*Triticum aestivum*) is a critical crop that provides a major source of calories to the global population [52]. Due to its essential role in food security, it is important to develop technology to explore allelic diversity and precisely control agronomic traits to produce robust varieties. As the population grows, the climate changes, and diseases develop and spread, the wheat germplasm needs to be enhanced to provide higher yields and withstand new environmental conditions and pathogens. Conventional breeding efforts have been imperative to this end [53], but the genetic complexity of the crop inflicts difficulty. The *Triticum aestivum* genome is approximately 17 Gb and allohexaploid (AABBDD), the result of hybridization events from three progenitor diploid species [54,55]. Because of this, most wheat genes exist as 3 homoeologous, but not necessarily identical, copies across the A, B, and D genomes. This redundancy and complexity due to allopolyploidy has made breeding and genetic manipulation in wheat challenging.

Gene editing by way of programmable target-specific nucleases has been revolutionary for precision breeding and functional genomics in wheat [6]. Clustered regularly interspaced short palindromic repeat associated protein 9 (Cas9), an RNA guided endonuclease [5], has emerged as a choice reagent due to its simple, modular, and precise gene editing system. Cas9, guided by RNA designed for sequence specificity, cleaves double stranded DNA. The host's endogenous repair mechanisms are triggered by the break, and non-homologous end joining, the dominant and often error-prone pathway in plants, can result in insertions or deletions at or near the site of cleavage and leads to new alleles or premature stop codons [56]. The process is of high value in wheat because systematic design of guide (gRNA) can conceivably allow for simultaneous mutation of all homoeologous genes, generating true gene knockouts in the allohexaploid genome. The delivery of DNA plasmids encoding Cas9 gene editing reagents to target wheat trait genes has been described using both *Agrobacteria* and particle bombardment [17-21].

Blast is a major agronomic disease of concern affecting wheat, rice, and other grass species. It is caused by the hemibiotrophic ascomycete fungus, *Magnaporthe oryzae*. Within the fungal species, there exists a high degree of host specificity [13]. *M. oryzae* that is pathogenic on rice and responsible for the destruction of 10-30% of global rice harvests [57], does not infect wheat [58]. In fact, wheat blast was first observed in 1985 in Paraná, Brazil [14,59] and subsequently spread through South America. In 2016, it was found in Bangladesh, and affected 15% of the country's wheat crop [59]. Wheat blast disease is caused by *Magnaporthe oryzae pathotype Triticum* (*MoT*) and is genetically distinct from, but closely related to, rice-infecting isolates [60]. It causes severe yield losses, posing an imminent threat to current and future food security. The utilization of Cas9 gene editing in wheat to reduce susceptibility to infection by *MoT* is promising.

Currently, few blast resistance (R) genes have been identified in wheat species [61]. These include Rmg8 and RmgGR119 [62,63], which can confer high resistance, but have been found to be overcome by a *MoT* effector gene [64]. The arms race between plant R genes and pathogenic effectors is a hallmark of plant-pathogen interactions. Because of this, breeding for R gene mediated disease resistance does not always provide durability. As an alternative, targeting negative regulators of resistance, or susceptibility (S) genes, for mutation or knock-out may be a viable method for obtaining blast disease resistance in wheat.

Genes conferring susceptibility to *M. oryzae* have been characterized in rice. One such gene is *Pi21* (Os04g0401000). The wild type gene codes for a proline-rich protein with a putative heavy metal binding domain. Two in-frame deletions give rise to a recessive allele, *pi21*, providing partial and race-nonspecific resistance to rice blast. RNAi mediated knockdown confers partial resistance, indicating that a knock-out may provide the same or perhaps increased resistance. The gene is closely linked to a locus responsible for poor taste, making it difficult to utilize via classic breeding [15]. Another negative regulator of resistance characterized in rice is *Bsrk-1* (Os10g0548200). The recessive allele, *bsr-k1*, identified from an ethyl methanesulfonate (EMS) mutagenized rice population, confers resistance as a result of early termination of a tetratricopeptide repeat (TPR) containing protein. The truncated protein fails to bind phenylalanine ammonia-lyase mRNAs, leading to an accumulation of those transcripts, subsequent enrichment of lignin, and increased broad spectrum resistance to both *M. oryzae* and *Xanthomonas oryzae*. Bsr-k1 knockout rice lines were also found to be resistant [16].

Here, we target putative wheat orthologs of the rice blast susceptibility genes, *Pi21* and *Bsrk-1* for Cas9-mediated gene editing. Given the family level phylogenetic relationship between rice and wheat, we reason that those orthologous genes may retain similar function. In recent work, driven by analogous reasoning, authors located putative wheat orthologs of two rice blast S genes, *Bsrd-1* and *Bsrk-1*. Using Targeting Induced Local Lesions IN Genomes (TILLING) by sequencing in an EMS population, they uncovered single nucleotide polymorphisms in the genes of interest that would lead to loss of function [65]. However, they did not generate plants with homozygous knockouts of either S gene. In the current study, we computationally identify putative wheat *Pi21* (*TaPi21)* and wheat *Bsrk-1* (*TaBsrk-1*) and design Cas9-based editing by plasmid particle bombardment to target them independently for full knockout in regenerable wheat immature embryos (IEs). We develop 3-plasmid and 1-plasmid gene editing schemes and describe an increase in editing efficiency by heat treatment of the bombarded IEs. We also present analysis of the distribution of editing depth across the 6 targeted homoeoalleles in each of 93 regenerated *TaBsrk-1* targeted plants, demonstrating the production of homozygous susceptibility gene knockouts across all three genomes of a wheat plant within the first (T_0) generation. Lastly, however, we find that homozygous disruptive mutations in all homoeologous copies of *TaPi21* or *TaBsrk-1* do not yield detectable resistance to infection by either *MoT* or *Xanthomonas translucens*.

3.2 Identification of *Pi21* and *Bsrk-1* wheat orthologs

To identify wheat orthologs of *Pi21* and *Bsrk-1*, we performed a BLAST search of the International Wheat Genome Sequencing Consortium (IWGSC) *Triticum aestivum* genome assembly via EnsemblPlants using rice *Pi21* (Os04g0401000/ LOC_Os04g32850) and rice *Bsrk-1* (Os10g0548200) as queries [15,16].

A previous study reported that no orthologs of Pi21 were identified in wheat [65], however, we identified putative orthologs in three wheat sub-genomes with new annotation version v2.1. These were TraesCS2A03G0723000.1 (*TaPi21A)*, TraesCS2B03G0799000LC.1 (*TaPi21B*), and TraesCS2D03G0666600LC.1 (*TaPi21D*), though two out of the three had low confidence of annotation. TraesCS2B03G0799000LC.1 was manually re-annotated for further analysis due to poor annotation in the reference. Using Clustal 2.1, a percent identity matrix was generated, revealing just under 60% amino acid sequence identity between the wheat and rice orthologs, and greater than 89% amino acid sequence identity between the A, B, and D subgenome homoeologs

(Table 2). Synteny analysis revealed that blocks with *Pi21* and *Pi21* orthologs were conserved among rice and the three wheat sub-genomes, even though the size of the blocks varied. We explored the genomic regions and found seven genes in rice and six genes in wheat (two genes in each sub-genome) that contained putative heavy-metal-transport/detoxification protein domains (Fig. 7). The domain sequences of the genes were conserved across all sequences (Fig. 8). A phylogenetic tree with these genes showed homologous relationships among the *Pi21* and *Pi21*-like genes (Fig 9).

Table 2: Percent identity matrix comparing amino acid sequence identity of *Pi21* orthologs

	OsPi21	***TaPi21A***	***TaPi21B***	***TaPi21D***
OsPi21	100	59.09	58.78	59.03
TaPi21A	59.09	100	89.64	92.19
TaPi21B	58.78	89.64	100	91.85
TaPi21D	59.03	92.19	91.85	100

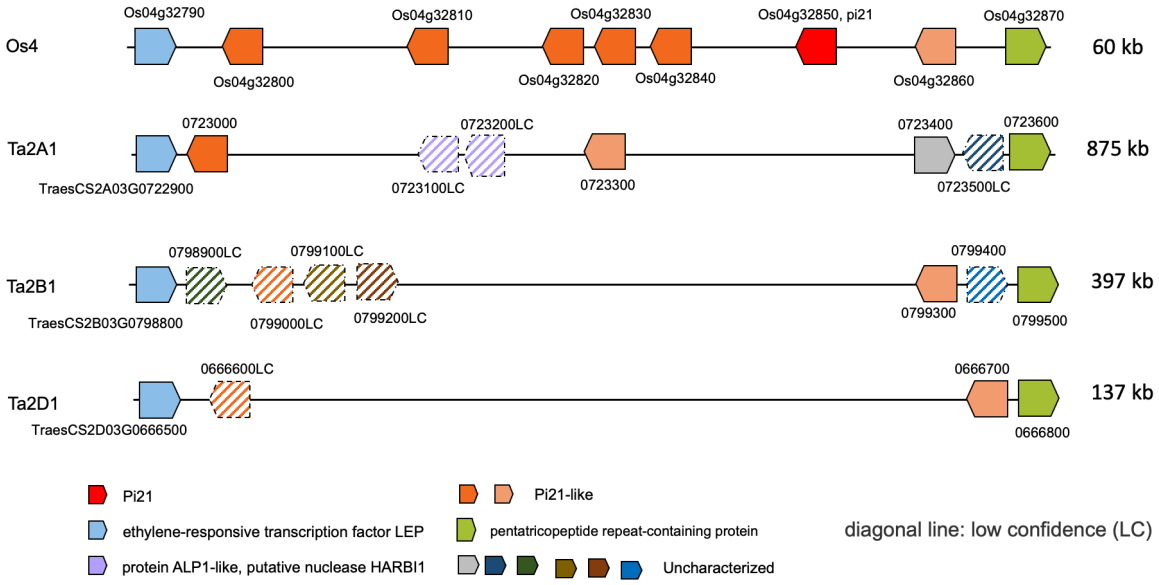


Figure 7. Synteny analysis of rice and wheat *Pi21* genomic regions.

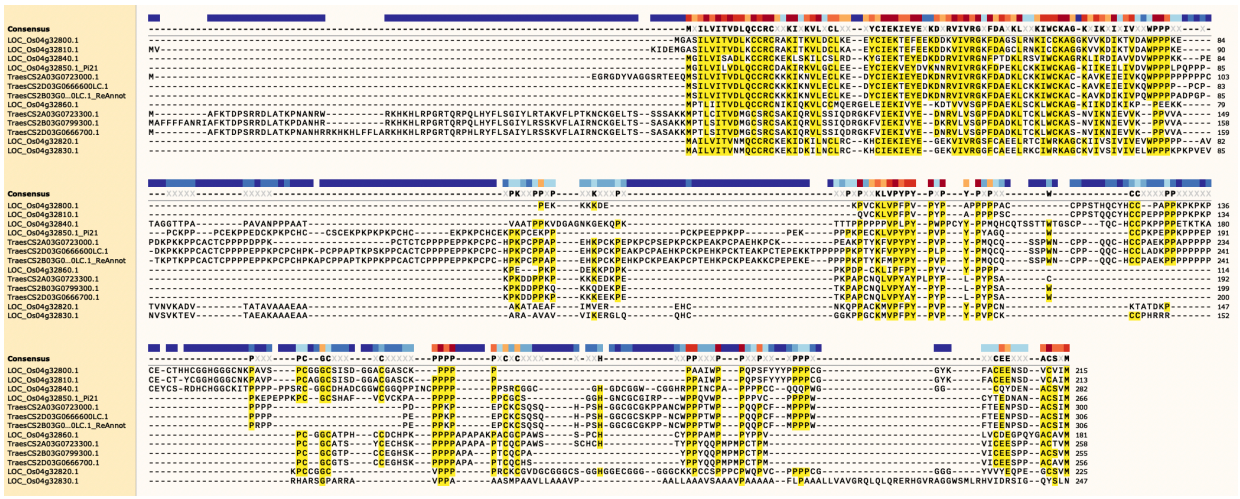


Figure 8. Multiple sequence alignment of *Pi21* orthologs

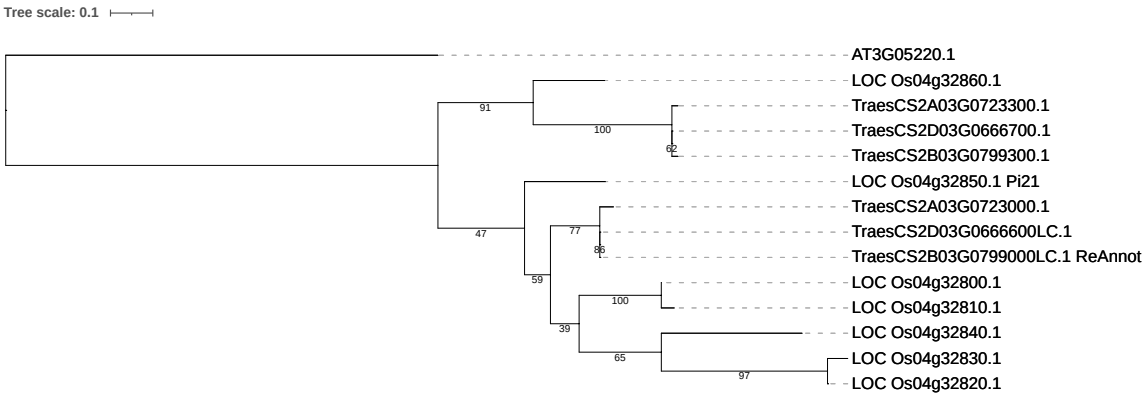


Figure 9. Phylogenetic tree of *Pi21* orthologs. The maximum likelihood tree was constructed with 500 bootstrap replications.

TraesCS1A02G207700 (*TaBsrk-1A*), TraesCS1B02G221400 (*TaBsrk-1B*), and TraesCS1D02G211000 (*TaBsrk-1D*) were identified as putative orthologs of rice *Bsrk-1*, consistent with previous findings [65]. Synteny analysis showed that regions containing *Bsrk-1* and *Bsrk-1* orthologs were conserved between rice and wheat (Fig. 10). Using Clustal 2.1, a percent identity matrix was generated, revealing approximately 80% amino acid sequence identity between the wheat and rice orthologs, and greater than 93% amino acid sequence identity between the A, B, and D subgenome homoeologs (Table 3). The pattern and number of TPR domains were conserved across all sequences. Taken together, these analyses indicated that we identified confident wheat orthologs of *Pi21* and *Bsrk-1*.

Table 3: Percent identity matrix comparing amino acid sequence identity of *Bsrk-1* orthologs

	OsBsrk-1	***TaBsrk-1A***	***TaBsrk-1B***	***TaBsrk-1D***
OsBsrk-1	100	81.55	78.55	80.37
TaBsrk-1A	81.55	100	93.92	97.22
TaBsrk-1B	78.55	93.92	100	93.49
TaBsrk-1D	80.37	97.22	93.49	100

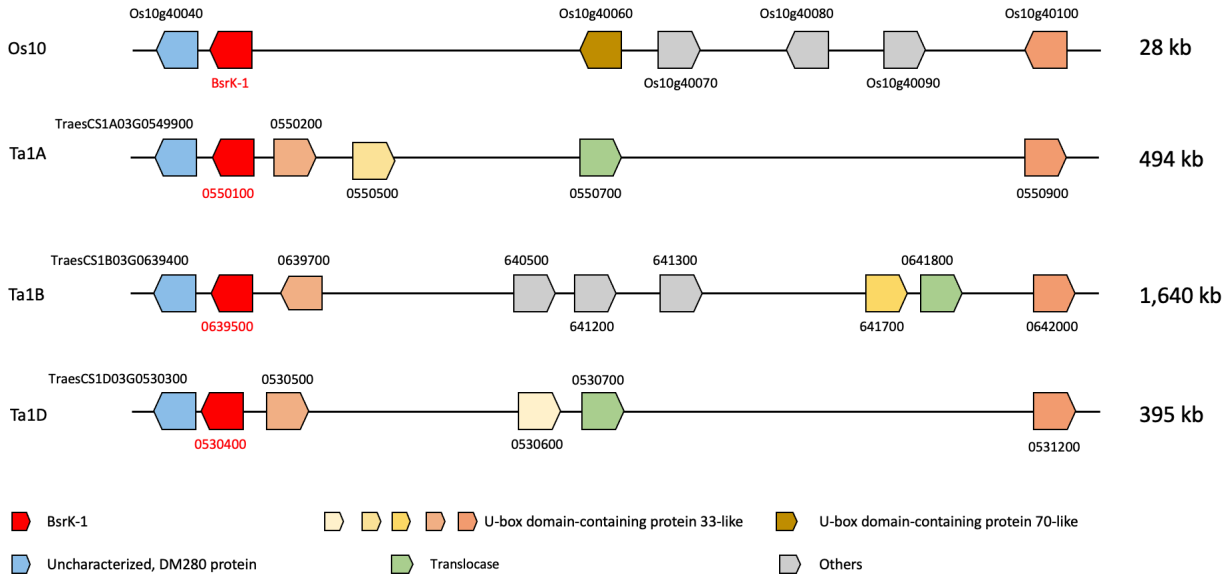


Figure 10. Synteny analysis of rice and wheat *Bsrk-1* genomic regions.

3.3 Generating mutations in *TaPi21*

We employed a three-plasmid biolistic approach for Cas9-mediated gene editing to generate site specific mutations in *TaPi21* homoeologs. To do so, we utilized the co-bombardment of three plasmids pAct1|HPT-4 [66], pJET1.2-OsUbi-Cas9-NosT, and pU6-gRNA[TaPi21gC+gD]. Stable expression of the construct pAct1|HPT-4 allowed for plant tissue culture selection on the antibiotic, hygromycin B. Meanwhile, Cas9 with two nuclear localization signals was expressed from pJET1.2-OsUbi-Cas9-NosT. Lastly, pU6-gRNA[TaPi21gC+gD] expressed two gRNAs, Pi21gC and Pi21gD, each designed to target all six alleles of *TaPi21*, in the second exon. The three

plasmids, mixed in equal amounts by weight, were precipitated onto gold microparticles and bombarded with high pressure into wheat immature embryos. These were maintained for regeneration under selection, and 11 resultant hygromycin resistant T_0 plantlets were transplanted to soil. Unfortunately, due to overheated greenhouse conditions, all but one T_0 plant was killed. The single surviving line, Pi21/60B, produced a mere five T_1 seeds from self-fertilization, which we subsequently planted. Remarkably, two of these T_1 plants, Pi21/60B-1 and Pi21/60B-5, had homozygous mutations in all three homoeologs of *TaPi21* (Table 4). In genome A for both lines, the +1 bp homozygous mutation caused a premature stop codon and a 54 amino acid truncation in the typically 283 amino acid protein. Although this is a significant truncation, it is not certain if the protein product was left nonfunctional by this mutation. In genome B for both lines, the homozygous mutation was a 493 bp deletion spanning from the Pi21gC cleavage site to 1 bp 3' of the Pi21gD cleavage site. It is highly likely that such a large deletion would leave the typically 306 amino acid genome B product nonfunctional. In genome D, for line Pi21/60B-1, the +1 bp homozygous mutation caused a premature stop codon resulting in a 159 amino acid truncation. In genome D, for line Pi21/60B-5, the -1 bp homozygous mutation caused a premature stop codon resulting in a 235 amino acid truncation. The wildtype protein product of *TaPi21D* is 308 amino acids. Lines Pi21/60B-1 and Pi21/60B-5 were followed to the T_2 and T_3 generations. The homozygous mutations generated from Cas9-mediated editing were maintained and detected in all plants.

Table 4: Summary of T_1 *Pi21* Cas9-targeted lines

		Genome A		**Genome B**		**Genome D**	
T1 Line	**Guide**	**Mutation**	**Type**	**Mutation**	**Type**	**Mutation**	**Type**
Pi21/60B-1	Pi21gC	+1	Homozygous	Deletion spanning guides	Homozygous	None	WT
	Pi21gD	None	WT			+2	Homozygous
Pi21/60B-2	Pi21gC	+1	Homozygous	Deletion spanning guides	Homozygous	None	WT
	Pi21gD	-3	Heterozygous			+1	Heterozygous
Pi21/60B-3	Pi21gC	None	WT	Deletion spanning guides	Homozygous	None	WT
	Pi21gD	+1	Homozygous			+1	Heterozygous
Pi21/60B-4	Pi21gC	+1	Homozygous	-2	Homozygous	None	WT
	Pi21gD	-3	Heterozygous	-1	Homozygous	+1	Heterozygous
Pi21/60B-5	Pi21gC	+1	Homozygous	Deletion spanning guides	Homozygous	None	WT
	Pi21gD	None	WT			-1	Homozygous

3.4 Generating mutations in *TaBsrk-1*

In contrast to our efforts in *TaPi21* we utilized a single plasmid, pCam-2xU6-gRNAef[TaBsrk1g8+g38], to edit *TaBsrk-1* homoeologs. The construct encoded hygromycin phosphotransferase for selection, Cas9 with two nuclear localization signals, and two gRNAs, TaBsrk1g8 and TaBsrk1g38, each designed to target all six alleles of *TaBsrk-1* in the first and fourth exon, respectively. Plasmid DNA was precipitated onto gold microparticles and bombarded with high pressure into wheat immature embryos. The bombarded embryos were divided into three

subsets. The first underwent the standard plant tissue culture process for regeneration. The other two were subjected to either 30°C or 37°C treatment at four days post-bombardment for 16 hours. In total, 93 regenerated T_0 plantlets were recovered from hygromycin selection and transplanted to soil.

3.5 Analysis of editing outcomes and temperature treatment in *Bsrk-1* targeted T0 regenerants

We utilized amplicon next generation sequencing (NGS) to genotype each of the 93 T_0 plants. Primers for PCR were designed to amplify *TaBsrk-1* sequences from all three subgenomes in a single reaction. Single nucleotide polymorphisms that are unique to each subgenome allowed for the deconvolution of data and assignment of the alleles detected to either the A, B, or D genome. Analysis revealed that TaBsrk1g8 yielded no Cas9 mediated editing in any of the regenerants. Therefore, all data presented here represent gene editing at the TaBsrk1g38 cleavage site. Analysis of the editing outcomes is summarized in Figure 11. More than 50% of the regenerated T_0 plants were found to contain edits in a minimum of one out of the six alleles targeted. Notably, 20% of the plants had homozygous or biallelic mutations in all three homoeologs, demonstrating that generating triple homozygous or biallelic mutants of allohexaploid wheat is feasible within the first generation. All mutations generated resulted in a premature stop codon in the fifth of twenty-two exons of *TaBsrk-1*. This termination results in a major truncation that eliminates a portion of the protein product's TPR domain. In addition to the analysis of total genotypes in this study, we further analyzed gene editing outcomes in *TaBsrk-1* based on temperature treatment (Figure 12). We found that treating the biolistically transformed IEs at 30°C or 37°C four days after bombardment for 16 hours yielded higher editing rates compared to the standard 26°C treatment.

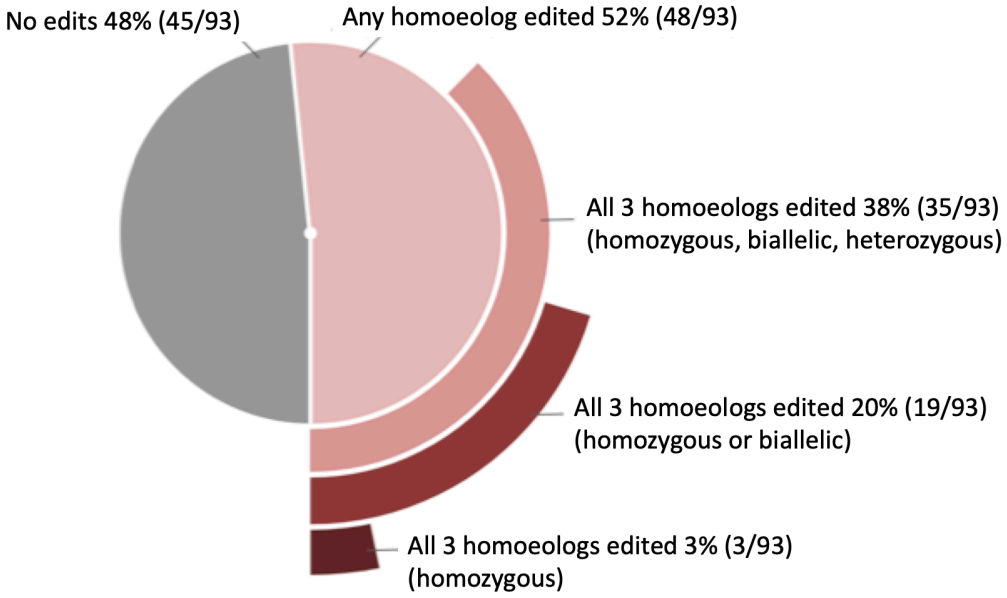
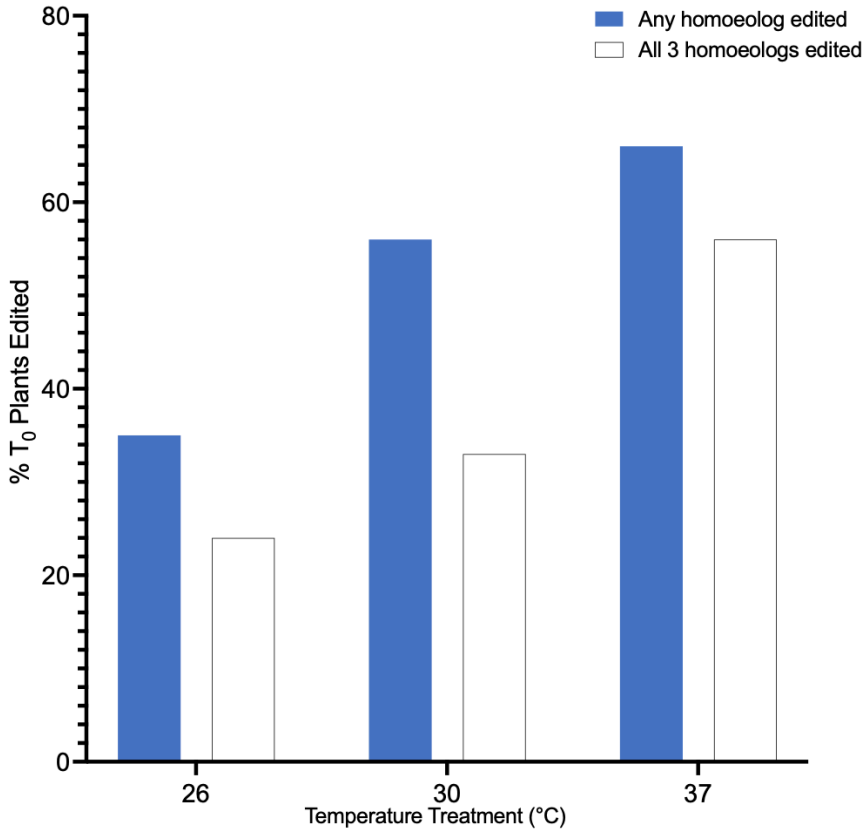


Figure 11. T_0 editing outcomes from *TaBsrk-1* targeting. Over half of all selected regenerants were edited in at least 1 of the 6 targeted alleles, 38% were edited in all 3 homeologs, 20% had homozygous or biallelic mutations in all 3 homeologs, and 3% had exclusively homozygous

mutations in all 3 homeologs. All Cas9 induced mutations resulted in a premature stop codon within the coding sequence, rendering the susceptibility gene nonfunctional.



	26°C	30°C	37°C
Any homoeolog edited	12/34 = 35%	15/27 = 56%	21/32 = 66%
All 3 homoeologs edited	8/34 = 24%	9/27 = 33%	18/32 = 56%

Figure 12. Analysis of *TaBsrk-1* editing based on temperature treatment. Of the 93 plants, subsets were regenerated from transformed embryos that were treated at 26°C, 30°C, or 37°C for 16 hours during recovery prior to callus induction. Only 37°C treated embryos gave rise to any plants with homozygous mutations in all three homoeologous genes. The data show a trend toward increased rate and breadth of editing in embryos treated at 37°C after bombardment compared to 26°C and 30°C treatments.

3.6 Knockout of *Pi21* or *Bsrk-1* wheat orthologs do not confer resistance to *Xanthomonas translucens* or *Magnaporthe oryzae pathotype Triticum*

In rice, loss of function mutation or RNAi knockdown of *Pi21* yielded incomplete but durable resistance to infection by *M. oryzae* [15]. Similarly, loss of function mutation or Cas9-mediated knockout of *Bsrk-1* led to broad spectrum resistance to both *M. oryzae* and *X. oryzae* [16]. Wheat pathogens that are analogous to rice-infecting *M. oryzae* and *X. oryzae* are *MoT* and the bacteria *Xanthomonas translucens*, respectively. To determine if triple homozygous mutations in

TaPi21 or *TaBsrk-1* could confer similar resistance, M_2 plants were subjected to inoculation by these pathogens.

The *X. translucens* line, M1(Tal2) [67], was inoculated by syringe infiltration into the lines M_2-Pi21/60B-1, M_2-Pi21/60B-5, and *TaBsrk*-1 triple homozygous mutant lines, M_2-Bsrk-1/18-10, and M_2-Bsrk-1/19-6, alongside wildtype Fielder wheat plants. We generated infection curves by determining the number of *X. translucens* colony forming units per square centimeter of inoculated leaf tissue at 0, 3, 7, 11, and 14 days post inoculation (Figure 13). There were no significant growth differences between the homozygous triple mutant lines and the wildtype control. Therefore, none of these mutant lines exhibited any level of resistance to infection by *X. translucens*.

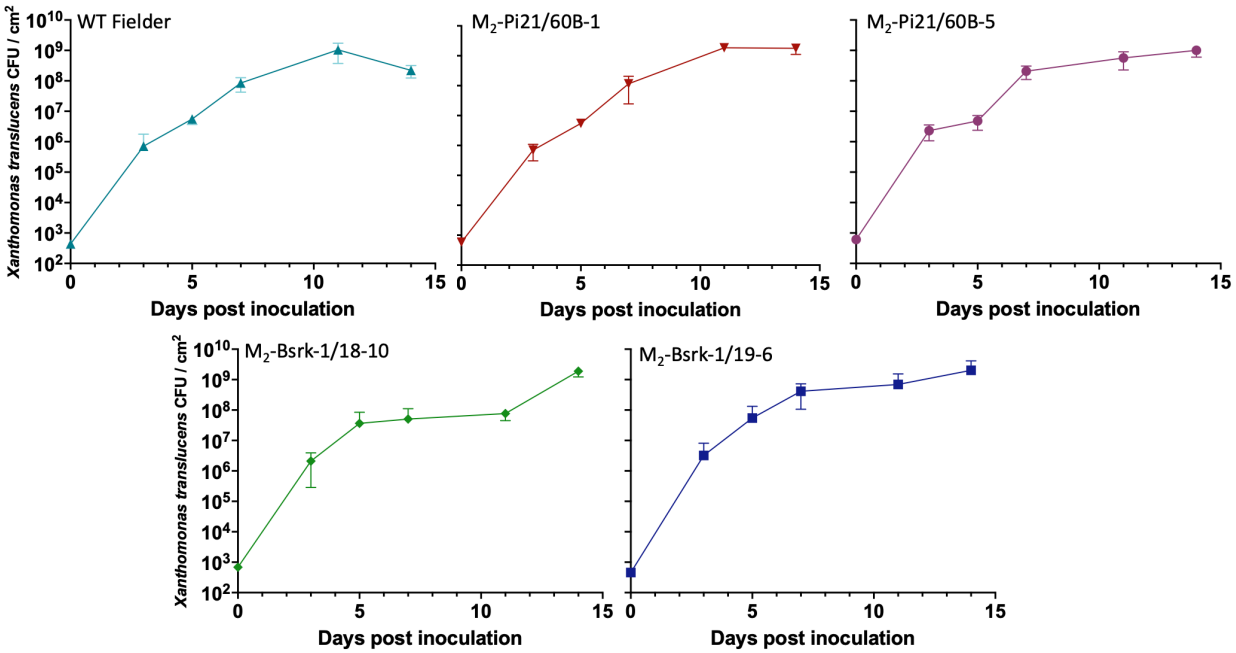


Figure 13. *Xanthomonas translucens* growth curves.

Inoculations to determine response to *MoT* infection were performed on the edited line M_2-Pi21/60B-1, its corresponding wildtype control Fielder, Bob White, a susceptible cultivar, and Urubo, a resistant cultivar. This work was carried out under biosafety level-3 (BSL-3) conditions at the Biosecurity Research Institute (BRI) in Manhattan-KS. Plants were randomized with two replications, with two to five heads inoculated per replicate. Disease severity average of the heads within a pot was considered as the replication and it was used in the statistical analyses. Three monosporic isolates were used for this experiment. Isolate T-25, collected in the state of Parana (Brazil) in 1988; isolate B-71, collected in Okinawa (Bolivia) in 2012; and isolate 16MoT001 collected in Passo Fundo – RS (Brazil) in 2016. T-25 was classified as race 1 and it is considered as a less-aggressive isolate; and isolates B-71 and 16MoT01 were classified as race 2 and are considered as aggressive isolates. The *MoT* isolate T-25, yielded the lowest disease severity, followed by B-71. The 16MoT01 isolate was significantly more aggressive on all genotypes compared to isolates T-25 and B-71. Despite the numerical differences between the mutant lines (M_2-Pi21/60B-1, M_2-Pi21/60B-5, M_2-Bsrk-1/18-10) and Fielder wildtype, these differences were not statistically significant ($p < 0.05$) for any of the three isolates (Table 5).

Table 5: Wheat head blast severity (%) for the Cas9-edited M_2 lines, Fielder wildtype, plus susceptible (Bob White) and resistant checks (Urubo) for each of the three isolates used.

MoT isolate	Wheat line	Disease severity %
T-25	Bob White	95.65
	Urubo	1.75
	M_2-Pi21/60B-1	62.9
	M_2-Bsrk-1/18-10	60.5
	M_2-Bsrk-1/19-6	56.8
	Fielder Wildtype	57.3
B-71	Bob White	91.25
	Urubo	9.0
	M_2-Pi21/60B-1	70.0
	M_2-Bsrk-1/18-10	73.5
	M_2-Bsrk-1/19-6	77.8
	Fielder Wildtype	76.5
16MoT01	Bob White	95.4
	Urubo	14.6
	M_2-Pi21/60B-1	86.5
	M_2-Bsrk-1/18-10	88.3
	M_2-Bsrk-1/19-6	90.2
	Fielder Wildtype	91.3

3.7 Discussion

In this study, we identified three homoeologous wheat orthologs each of the rice blast susceptibility genes, *Pi21* and *Bsrk-1*. We generated a full array of edited genotypes, including triple homozygous and biallelic knockout lines by using plasmid bombardment-based Cas9-mediated gene editing on wheat IEs to target the homoeologous genes. We also demonstrated that increased temperature treatment leads to higher rates of editing.

In addition to the challenges of suboptimal greenhouse conditions, the limitations of the three-plasmid approach to generate edits in *TaPi21* were clear. Because the gene editing reagents and hygromycin selection gene all existed independently on separate DNA constructs, heavy

reliance was placed on successful co-delivery of all three plasmids into the same embryonic cell, without verification checkpoints throughout the experiment. This led us to the single-plasmid gene editing approach to generate *TaBsrk-1* mutants, in which the single construct encoded the gene editing reagents, Cas9 and gRNA, and the hygromycin resistance cassette. We chose to regenerate almost 100 plants and systematically genotype every T_0 regenerant. More than 50% of the plants contained edits in at least one allele of at least one *TaBsrk-1* homeolog. Moreover, 20% of the regenerated plants contained homozygous or biallelic mutations in all three homoeologous *TaBsrk-1* genes. This result is significant because it demonstrates that this method can be used to generate fully homozygous triple knockouts in allohexaploid wheat and eliminate the need for any additional crosses. Our results showing increased editing efficacy of higher temperature treatment is consistent with prior studies [68,69], further solidifying the idea that Cas9, an endonuclease derived from bacteria with optimal physiological temperature of 37°C, activity in plants is improved at temperatures approaching 37°C.

Finally, we found that our Cas9-mediated triple homozygous knockouts of *TaPi21* or *TaBsrk-1* did not confer resistance to infection by *Magnaporthe oryzae pathotype Triticum* or *Xanthomonas* species despite what was described in the knockouts of the rice orthologs [15,16]. This result is, perhaps, unsurprising since the wheat genome, at 17 Gb, is 40x larger than the 389 Mb rice genome. The large, allohexaploid wheat genome may potentially contain functional redundancy of *TaPi21* and *TaBsrk-1*. Therefore, a full knockout of the gene may still be complemented by protein products with analogous function. Also, given divergent evolution of *MoT* from rice-infecting *M. oryaze*, there may be genetic differences governing virulence and pathogenicity. These results suggest that identifying wheat *MoT* resistance based on knowledge associated with rice blast resistance is not necessarily robust, and a deeper understanding of the *MoT*-wheat interaction is required.

3.8 Materials and Methods

Plant material

Triticum aestivum L. cv. Fielder, *Triticum aestivum* L. cv. Urubo, *Triticum aestivum* L. cv. Fielder, and *Triticum aestivum* L. cv. Bobwhite were used for this study.

Sequence analysis of Pi21 and Bsrk-1 orthologs in rice and wheat

The protein sequences were aligned with MAFFT and the alignment of the domain was used to construct a maximum-likelihood tree using RAxML with 500 bootstrap replications. Annotation information of rice (RGAP v7) and wheat (iwgsc_refseqv2.1) were used for synteny analysis using MCScanX. The gff information of the genes were extracted and used to illustrate the synteny blocks.

Plasmid constructs

Plasmids, pAct1IHPT-4, pAct1IDsRED, pJET1.2-OsUbi-Cas9-NosT, pU6-gRNA[TaPi21gC+gD], and pCam-2xU6-gRNAef[TaBsrk1g8+g38] were used.

pAct1IHPT4 [66] and pAct1IDsRED contain hygromycin phosphotransferase (hpt) and DsRED genes, respectively, each under control of the rice actin 1 promoters, its intron (act1I) and the nopaline synthase 3' terminator.

The backbone pJET1.2/blunt and CloneJET PCR Cloning Kit (ThermoFisher, Waltham, MA) were used to create pJET1.2-OsUbi-Cas9-NosT, containing Cas9 with N-terminal 3X FLAG,

SV40 NLS, C-terminal nucleoplasmin NLS and FLAG, regulated by the promoter of the rice polyubiquitin gene RUBQ2 and the nopaline synthase 3' terminator.

Gold particle preparation for bombardment

Plasmid DNA was precipitated onto 0.6 µm gold particles (#1652262, Bio-Rad, Hercules, CA) using $CaCl_2$ and spermidine. Briefly, 2 mg sterile gold particles were mixed with 5 µg of DNA in a total volume of 30 µl. To precipitate the DNA, 25 µl 5 M $CaCl_2$ and 20 µl 0.1 M spermidine were added and incubated on ice for 10 min. Samples were centrifuged at 14K rpm for 2 min, and the supernatant removed. Gold particles were rinsed with 500 µl cold 100% EtOH and incubated on ice for another 10 min, then centrifuged and the supernatant removed. The samples were resuspended in 85 µl 100% EtOH and distributed in 7.5 µl aliquots across the center of 10 macrocarriers. The gold particle covered macrocarriers were dried in a laminar flow hood. Each macrocarrier was used for a single bombardment.

Immature embryo bombardment and regeneration

Plants were grown at 24°C, 16-hour days and 15°C, 8-hour nights under light intensity of 130 µmol $m^{-2}s^{-1}$. Immature seeds containing IEs, sized 1.7-2.2 mm were harvested from wheat spikes 10-13 days after flowering, surface sterilized in 20% (v/v) bleach with one drop of Tween 20 and triple rinsed with sterile water, followed by extraction of the IEs. The IEs were placed on DBC3 media [66], scutellum side up and incubated overnight at 26°C prior to biolistic transfection. Four hours prior to bombardment, IEs were placed on 40 mm filter paper in the center of DBC3 osmoticum media containing 0.2 M mannitol and 0.2 M sorbitol [49]. Using two prepared microcarriers holding plasmid DNA coated gold microparticles, IEs were shot twice using the PDS-100/He gene gun (Bio-Rad, Hercules, CA) with rupture pressure of 650 psi. The bombarded IEs were transferred from the filter paper directly to the media below and incubated at 26°C 16 hours. IEs were transferred to standard DBC3 media in dim light (10 µmol $m^{-2}s^{-1}$) at 26°C for 4 days, followed by incubation at 26°C (standard), 30°C, or 37°C for 16 hours. Plates were returned to 26°C for an additional 3 days of rest. Following the resting period, embryos were placed on DBC3 media containing 30 mg/l hygromycin B for selection, with 3 rounds of weekly subculturing. Callus tissue originating from each IE was transferred to DBC6 media [70]. Resultant plantlets were transferred to rooting media and incubated in high light (90 µmol $m^{-2}s^{-1}$) at 26°C and grown to 4-6 inches before being transplanted to soil.

Genotyping by amplicon next generation sequencing

To analyze genotypes by amplicon sequencing, PCR was performed with target-specific primers on genomic DNA obtained by CTAB extraction, amplifying approximately 225 bp around the cut site using Phusion High Fidelity (New England Biolabs, Ipswich, MA) polymerase. Primers contained a 5′-stub compatible with Illumina NGS library preparation. PCR products were ligated to Illumina TruSeq adaptors and purified. Libraries were prepared using a NEBNext kit (Illumina, San Diego, CA) according to the manufacturer's guidelines. Samples were deep sequenced on an Illumina MiSeq at 2x300 bp paired-end reads to a depth of 10,000 – 100,000 reads per sample. Cortado (https://github.com/staciawyman/cortado) was used to analyze editing outcomes. Briefly, reads were adapter trimmed then merged using overlap to single reads. These joined reads were then aligned to the A, B, and D subgenome target reference sequences.

MoT inoculum production, inoculation, and disease rating

Inoculum production, inoculation procedure, and disease rating followed protocol previously described[71].MoT cultures were grown on homemade oatmeal agar [72] and when sporulating cultures were 8-days old, they were flooded with a mixture of sterile deionized water with 0.42% unflavored gelatin and 0.01% Tween 20, stirred to dislodge conidia from conidiophores, and filtered with four layers of sterile cheesecloth to eliminate mycelium and any remaining agar medium. The spore concentration was adjusted to 20,000 spores ml^{-1} of suspension. An amount of 0.75 ml of the spore suspension was individually applied to each wheat head approximately 2 days after full head emergence (Feekes GS 10.5, Large 1954) using an airbrush (model 69492, Harbor Freight Tools, Camarillo, CA). To provide the optimal conditions for MoT infection, the heads were covered with black plastic bags (model S-12322BL, ULINE) moistened with water for 48 hours immediately following spore suspension application. Disease severity was evaluated every other day, starting at eight until 16 days after inoculation, where each head was individually rated for the percentage of affected spikelets in a 0 to 100% scale. Disease severity was assessed on multiple days to characterize disease progress. However, for statistical purposes, we used the data collected when the susceptible check reached 90% or more of disease severity, which was different for each isolate. Data were analyzed using SAS Version 9.2 (SAS Institute, Cary, NC, 2001). We analyzed the disease severity evaluated at 10 days for isolate T-25 and at 8 days for isolates B-71 and 16MoT01 (i.e., susceptible check averaged 90% or more of disease severity) after inoculation using PROC GLIMMIX.

CHAPTER 4. Impact of temperature and time on DNA-free Cas9-ribonucleoprotein mediated gene editing in wheat protoplasts and immature embryos

4.1 Introduction

Amidst a rapidly growing population and threats posed by climate change and disease, there exists a need for the advancement of crop biotechnology to increase the speed and precision of crop varietal development. Cas9 has emerged as a plant gene editing tool of choice for its accuracy and programmability to engineer allelic diversity for beneficial traits to support global food security. Guided by RNA, Cas9 efficiently makes sequence-specific double-stranded breaks in genomic DNA [5]. The host's double-stranded break repair mechanisms are then elicited. Non-homologous end joining (NHEJ), the predominant and often error prone pathway in plants, can lead to insertions or deletions (indels) at the Cas9 cut site upon repair [56]. Exploitation of this system allows for targeted knockout of endogenous genes.

Cas9 and guide RNA (gRNA) encoding plasmid DNA systems have been developed and delivered to plant and major crop species including *Arabidopsis* [73], potato [74,75], tomato [76,77], soybean [78], maize [79,80], barley [81,82], rice [83], and wheat [17] by *Agrobacterium tumefaciens* or particle bombardment. These methods rely on random integration of Cas9-gRNA cassettes into the genome, and optimization of expression for each plant system. As a result, the gene editing process is encumbered by variables such as promoter and terminator choice when cloning constructs and copy number and integration location of transgenes upon transformation. Additionally, gene editing by these methods raise transgenic regulatory concerns. Regulation aside, transgenes can often be segregated away through breeding, but the process is laborious, time consuming, and particularly difficult for plants with complex genomes. Moreover, crops with lengthy generation times or those that are vegetatively propagated, such as cassava and banana, cannot be bred to segregate transgenes. There have been reports in which plant gene editing has been achieved by transient expression of Cas9 and gRNA [84,85], however full experimental control over the fate of transgene integration and tracking has not been achieved. For these reasons, there is a clear need for advances in DNA-free genome editing technology.

The direct delivery of preassembled Cas9-gRNA ribonucleoproteins (RNPs) is one such technology and has been demonstrated in various plant protoplast systems to induce targeted mutations [51,86-89]. Some have produced edited plants arising from the transfected single cells. However, regeneration of wheat and other crop plant protoplasts is not feasible with current methods. Cas9-RNP based editing of maize [22], rice [23], and wheat [24] regenerable embryos by biolistics has also been reported. Gold particles coated with Cas9-RNPs are bombarded with high pressure into immature embryos (IEs) that are ultimately regenerated into plants through tissue culture. Co-delivery of DNA vectors with selective markers or helper genes along with Cas9-RNPs have been utilized to improve editing efficiency [22,23]. In the absence of selection, however, editing rates have generally been low.

The use of Cas9-RNPs to generate edited plants provides unique benefits. Because the gene editing reagents are delivered as pre-assembled complexes, researchers do not need to optimize DNA vectors, the host plant tissue does not bear the burden of transcribing or translating Cas9 or gRNA, and breeding for segregation is unnecessary due to the absence of transgenes. Additionally, the Cas9-RNPs, which exist in a finite amount in the target tissue, are ultimately degraded by endogenous proteases and nucleases. However, there remains room to improve the editing pipeline and increase efficiency.

Low rates of Cas9 mediated editing in plant tissue may indicate that the endonuclease is not reaching its full potential due to suboptimal environmental conditions. For example, studies across organisms including Arabidopsis, citrus [68], and wheat [69] have shown that Cas9 generates more targeted indels at elevated temperatures.

Here, we present advances in Cas9-RNP based gene editing in the global food crop, wheat. To determine if temperature can be harnessed to enhance Cas9-RNP mediated editing, we explore the effects of heat treatment on transfected wheat protoplasts and IEs. We examine the relationship of editing efficiency between non-regenerable protoplasts and regenerable IEs and monitor the rate of editing over time. We demonstrate that treatment at elevated temperatures increases gene editing efficiency in both tissue systems and find that the RNP transfection technique of gold particle bombardment results in sustained editing of tissue at least 14 days after bombardment. We also find that editing rates in protoplasts correlate linearly with editing rates in IEs. Therefore, rapid *in vivo* protoplast assays can be instituted as a standard gene editing pipeline step to select the most effective gRNAs for IE gene editing and regeneration. Lastly, we regenerate wheat plants edited via Cas9-RNP biolistic transfection. As a proof of method, we simultaneously target three wheat homoeologous orthologs of a rice gene, *Pi21*(Os04g0401000), and successfully generate lines with knockouts in all copies. We also target wheat genes *Tsn1* and *Snn5*, producing lines that are insensitive to the *Parastagonospora nodorum* pathogenic effectors SnToxA and SnTox5 and establish DNA and selection-free Cas9-RNP mediated editing as an efficient and feasible technique for generating targeted gene knockouts in wheat.

4.2 Cas9-RNP transfection and the effect of temperature in wheat protoplast gene editing

We first quantified cell viability after heat treatment of non-transfected protoplasts to determine the feasibility of testing higher temperatures for wheat protoplast gene editing. Protoplasts were isolated from partially etiolated wheat seedlings and incubated at 25°C, 30°C, or 37°C for 16 hours followed by 25°C for 8 hours. During the 24-hour period, the protoplasts were monitored for viability every 8 hours using Evans blue staining and microscopy. Viability of protoplasts treated at 37°C decreased markedly compared to those treated at 25°C and 30°C (Fig. 14) and suffered from media evaporation. It was therefore concluded that the protoplast gene editing pipeline was not amenable to a 37°C heat treatment.

Five single guide RNAs (sgRNAs), Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were selected and commercially synthesized for this study. To assess the efficacy of the sgRNAs *in vivo*, and to determine the effect of temperature on wheat protoplast gene editing, Cas9-RNPs were assembled and transfected into wheat mesophyll protoplasts. Purified Cas9 with a C-terminal double nuclear-localization tag was complexed with sgRNA. The resulting sgRNA-Cas9 RNPs were transfected into wheat protoplasts using polyethylene glycol (PEG). Transfected protoplasts were treated at 25°C or 30°C and harvested for genotypic analysis after 24 hours. Editing rates at the target loci were determined by amplicon next-generation sequencing (NGS). With incubation at 25°C and 30°C, average editing rates ranged from 2.5-50% and 5.8-62% respectively. Despite this variability between different sgRNA-Cas9 RNPs, editing efficiency was consistently higher in protoplasts treated at 30°C compared to 25°C for any given sgRNA (Fig. 15), suggesting that a higher temperature treatment is advantageous to RNP-mediated gene editing in wheat protoplasts.

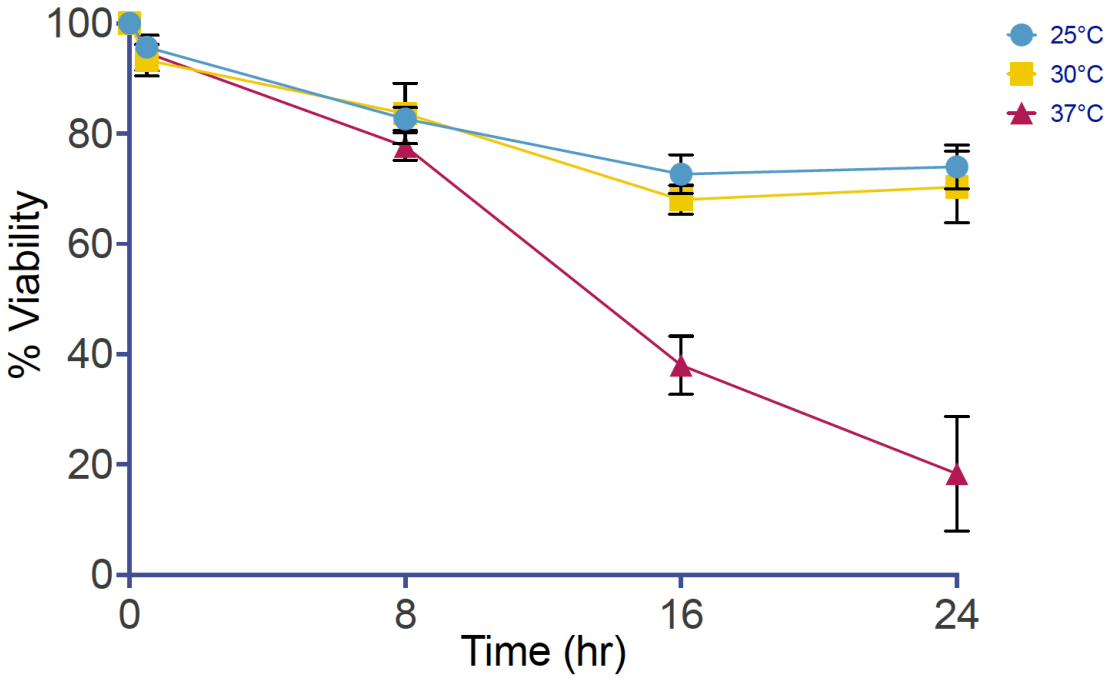


Figure 14. Protoplast viability curve. N=3. Error bars indicate SEM.

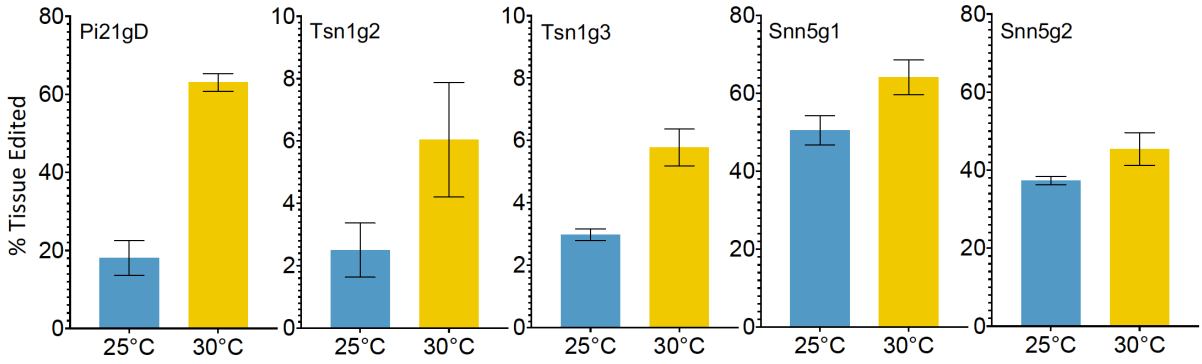


Figure 15. Targeted editing efficiency of gRNA-Cas9 ribonucleoproteins with different temperature treatments in wheat protoplasts. Five gRNAs, Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were tested and transfected independently into protoplasts. N=3. Error bars indicate SEM.

4.3 Biolistic Cas9-RNP delivery and the effect of temperature in wheat immature embryo gene editing

To determine if a high temperature treatment similarly improves Cas9-RNP based editing in wheat IEs as it does in protoplasts, RNPs were transfected into IEs by particle bombardment. The experimental pipeline is summarized in Figure 16a. Single guide RNA and Cas9 were complexed *in vitro*, adsorbed onto 0.6 µm gold particles, and biolistically delivered with a helium-pressured particle gun. For each sgRNA and temperature being tested, 30 IEs were bombarded and

incubated at 26°C, 30°C, or 37°C for 16 hours. They were then maintained at 26°C on callus-induction media before inducing regeneration at around 63 days post-bombardment (dpb). Plasmid DNA was not co-delivered with any of the Cas9-RNPs, and callus induction and regeneration were performed under selection-free conditions. From each set of 30 RNP-transfected embryos, ten were randomly harvested and pooled for genomic analysis at 14 dpb and again at 48 dpb. The remaining ten embryos were kept for regeneration into M_0 plants. All independent shoots were isolated and treated as individual M_0 plants. Plants were transplanted from tissue culture media to soil approximately 100 dpb. Each resulting M_0 plant was independently genotyped, and the percent tissue edited rate was calculated as the percentage of mutant alleles among total alleles in the M_0 plant pool. The percentage of plants edited was also calculated as a percentage of the number of plants with any edit among the number of total M_0 plants regenerated. All genomic analysis was done by amplicon NGS.

Elevated temperature treatment of both 30°C and 37°C led to higher percentages of edited tissue compared to 26°C for all five sgRNA-Cas9 RNPs across all timepoints (Fig. 16b). Tissue editing rates were higher at 48 dpb than at 14 dpb and editing rates in the M_0 regenerant tissue pool were comparable to those at 48 dpb. From the ten embryos per treatment allowed to regenerate, 10-40 M_0 plants were produced. Plants with wild type, heterozygous, biallelic, and homozygous mutations at the target loci were obtained. Editing efficiency in the M_0 regenerants is summarized in Table 6.

Table 6: Summary of editing outcomes in *Pi21, Tsn1*, and *Snn5* targeted M_0 plants. Data for *Pi21* is broken down by subgenome. *Tsn1* and *Snn5* are only present on subgenome B. "% Tissue Edited" indicates the percentage of edited alleles among the total alleles analyzed from the M_0 pools. "% Plants Edited" indicates the percentage of plants with any level of editing among the total plants analyzed from the M_0 pools.

					Genome A		Genome B		Genome D			
	Temperature Treatment	Immature embryos regenerated	Total M0 Plants	Edited Plants	Heterozygous	Biallelic or Homozygous	Heterozygous	Biallelic or Homozygous	Heterozygous	Biallelic or Homozygous	% Tissue Edited	% Plants Edited
Pi21gD	26°C	10	4	1	0	1	1	0	1	0	16.7	25
	30°C	10	4	2	0	1	0	1	1	1	29.2	50
	37°C	10	5	2	0	1	1	1	1	1	26.7	40

	Temperature Treatment	Immature embryos regenerated	Total M0 Plants	Edited Plants	Heterozygous	Biallelic or Homozygous	% Tissue Edited	% Plants Edited
Tsn1g2	26°C	10	20	1	1	0	2.5	5.0
	30°C	10	22	3	1	2	11.4	13.6
	37°C	10	40	7	6	1	10.0	17.5
Tsn1g3	26°C	10	32	4	4	0	6.3	12.5
	30°C	10	17	2	0	2	11.8	11.8
	37°C	10	20	3	2	1	10.0	15.0
Snn5g1	26°C	10	19	5	5	0	13.2	26.3
	30°C	10	16	6	2	4	31.3	37.5
	37°C	10	10	3	2	1	20.0	30.0
Snn5g2	26°C	10	10	1	1	0	5.0	10.0
	30°C	10	14	4	1	3	25.0	28.6
	37°C	10	18	5	3	2	19.4	27.8

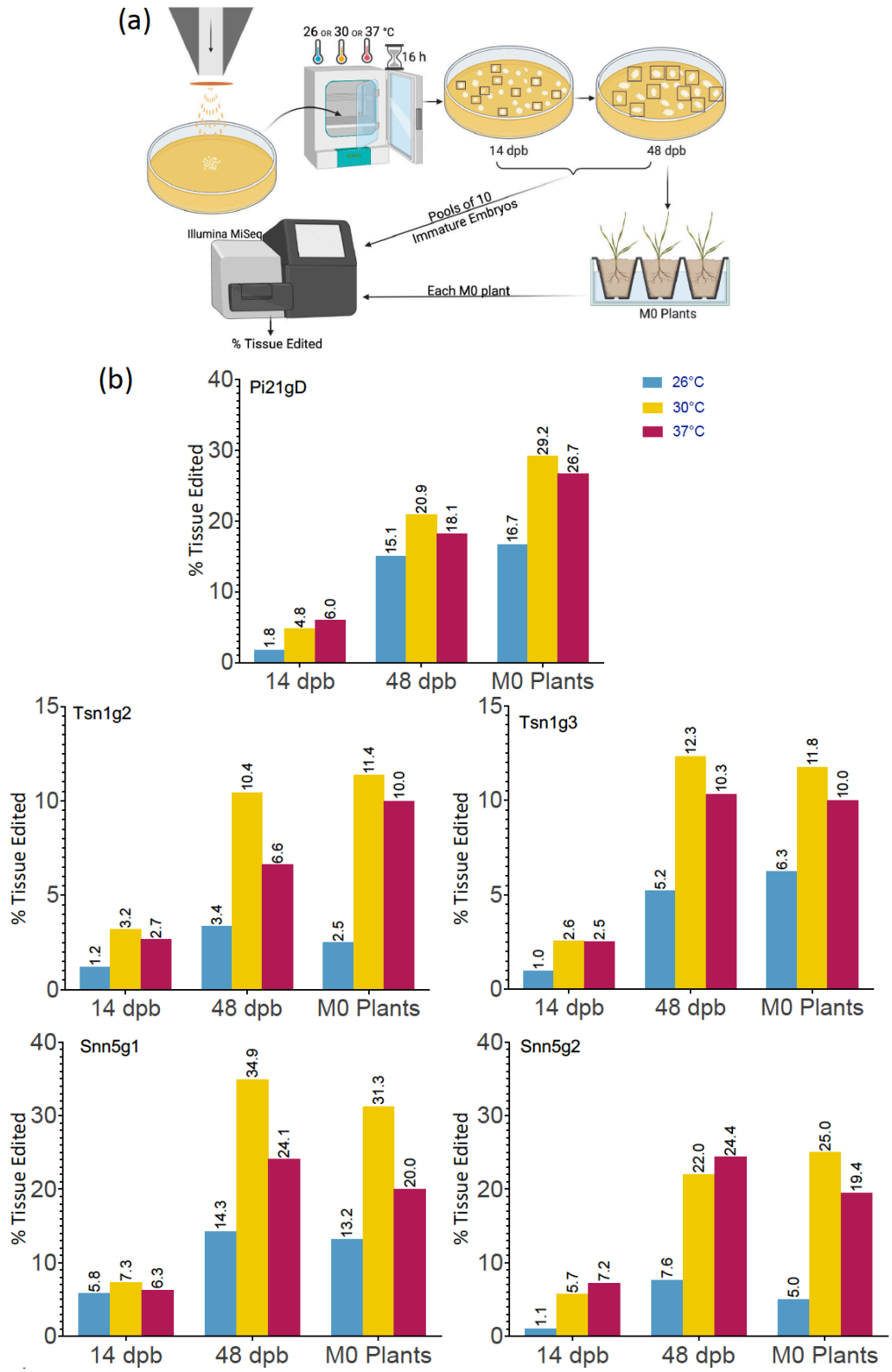


Figure 16. Cas9-RNP particle bombardment and temperature treatment of wheat immature embryos (IEs). (a) A schematic of the particle bombardment and editing efficiency assay pipeline. (b) Targeted editing efficiency of gRNA-Cas9 ribonucleoproteins with different temperature treatments in IEs across time points. Five gRNAs, Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were bombarded

independently into IEs. Tissue pools at 14 dpb and 48 dpb consisted of 10 randomly chosen initially bombarded IEs. Editing efficiency for M_0 plants is based on aggregate data from all independently genotyped M_0 plants that emerged from 10 randomly chosen initially bombarded IEs. Percent tissue edited is defined as the percentage of tissue with insertions or deletions within 2 bp of the target cleavage site out of the total tissue pool.

4.4 Cas9-RNP mediated editing is sustained over time

Notably, gene editing rates were more than doubled, regardless of temperature treatment, in tissue assayed at 48 dpb compared to 14 dpb (Figure 16b). To further investigate the difference in editing rates over time, the number of unique mutant alleles was determined at the 14 and 48 dpb timepoints. With minimal exception, there were more unique mutant alleles at 48 dpb compared to 14 dpb (Figure 17a, Figure 18).

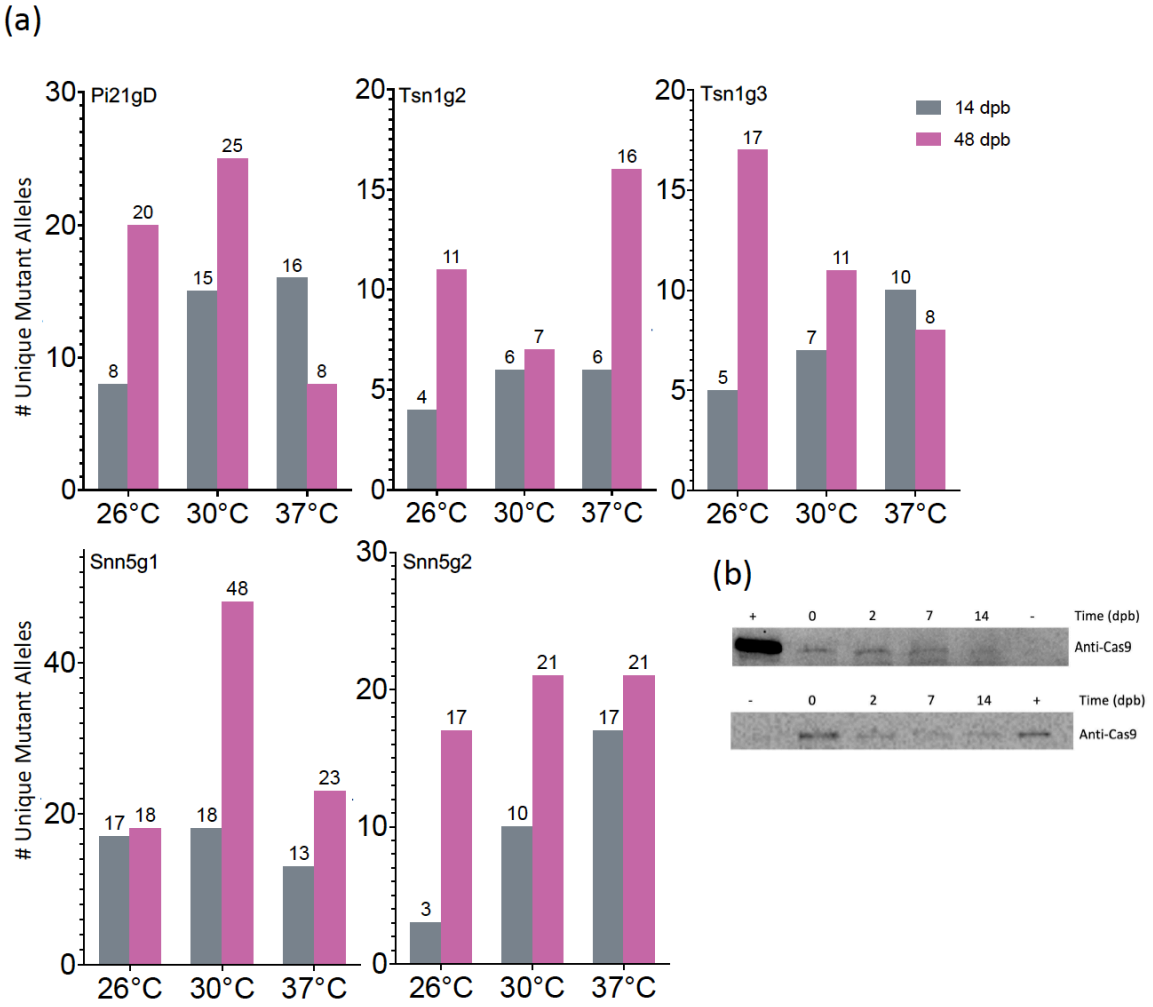


Figure 17. Cas9-RNP mediated editing in gold particle bombarded immature embryos IEs is sustained over time. (a) Quantification of the number of unique mutant alleles detected via deep sequencing. (b) Western blot detection of Cas9 in 10-IE bombarded samples taken 0, 2, 7, and 14 dpb with anti-Cas9 antibody. The top and bottom blot represent 2 independent sets of 10 IEs. + = 9 ng (top) and 3 ng (bottom) Cas9; - = IEs that were not bombarded with Cas9-RNP; loading volume of 25 µl (top) and 40 µl (bottom) total soluble protein extract per IE sample.

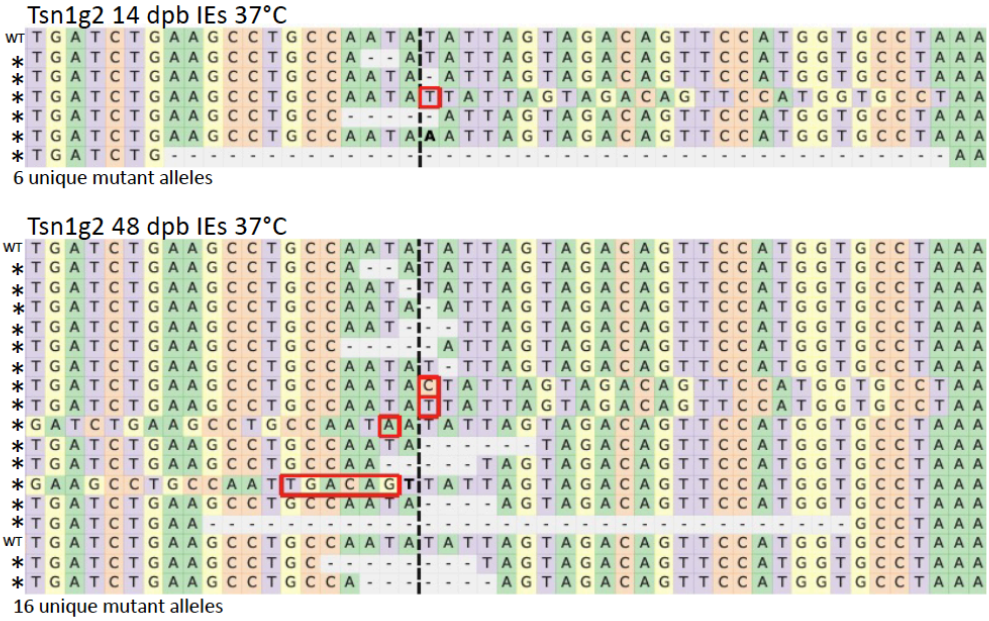


Figure 18. Example of the difference in the number of unique mutant alleles between 14 dpb and 48 dpb. Provided are the detected alleles in immature embryos bombarded with Tsn1g2-Cas9 RNPs and treated at 37°C. The vertical bold dashed line represents the Cas9 cleavage site. Mutant alleles are marked with *. Wild type alleles are marked as WT. Dashes indicate base pair deletions, red boxes indicate base pair insertions, and bold letters indicate base pair substitutions.

An additional 50 IEs were bombarded with Snn5g1-Cas9 RNP to determine the length of time that Cas9 remains present in biolistically transfected tissue. Western blot analysis was performed with 10-embryo tissue samples taken 0, 2, 7, and 14 dpb. Given the finite amount of Cas9 protein delivered by RNP bombardment and rapid cell division and growth in each IE over time, we normalized the experiment by volume extracted from total tissue originating from ten IEs at any given timepoint, rather than total protein extracted. Cas9 was detected in tissue from all four timepoints with decreasing band intensity over time (Figure 17b). Cas9 was not detected in embryos that were not subjected to bombardment of Cas9-RNPs. Due to the large mass of tissue from exponential growth of callus from IEs, it was not feasible to extract protein from and perform Western blot analysis on ten-embryo 48 dpb samples. Taken together, these results suggest that Cas9 mediated editing activity is sustained over the course of at least 14 days after biolistic delivery of Cas9-RNPs into immature wheat embryos. When using this method, the degradation of Cas9 protein in the target tissue is not as rapid as previously hypothesized [25], and evaluation of editing efficiency should occur 14 to 48 dpb for increased accuracy.

4.5 Relative editing rates in protoplasts correlate linearly with editing rates in M_0 regenerants from bombarded immature embryos

The different sgRNA-Cas9 RNPs used in this study conferred different levels of efficacy in both PEG transfected protoplasts and biolistically transfected embryos. To determine whether the editing rates in the two tissue systems correlated with one another, each sgRNA-Cas9 RNP's average editing efficiency in 30°C treated protoplasts was plotted against its editing efficiency in

48 dpb 30°C treated bombarded IEs as well as the M_0 30°C treated regenerant tissue pool. A linear regression model was applied to the data, revealing a positive linear correlation with R^2=0.744 and R^2= 0.994, respectively (Fig. 19). Though a survey of a greater number of sgRNAs would strengthen this association, the present data suggest that editing efficiency in protoplasts can be predictive of editing efficiency in IEs. Given the positive correlation between RNP-mediated editing rates in protoplasts and in biolistically transfected IEs, it can be beneficial to first rapidly score the efficiency of various gRNA candidates in protoplasts to optimize for the highest rate of edited regenerant tissue.

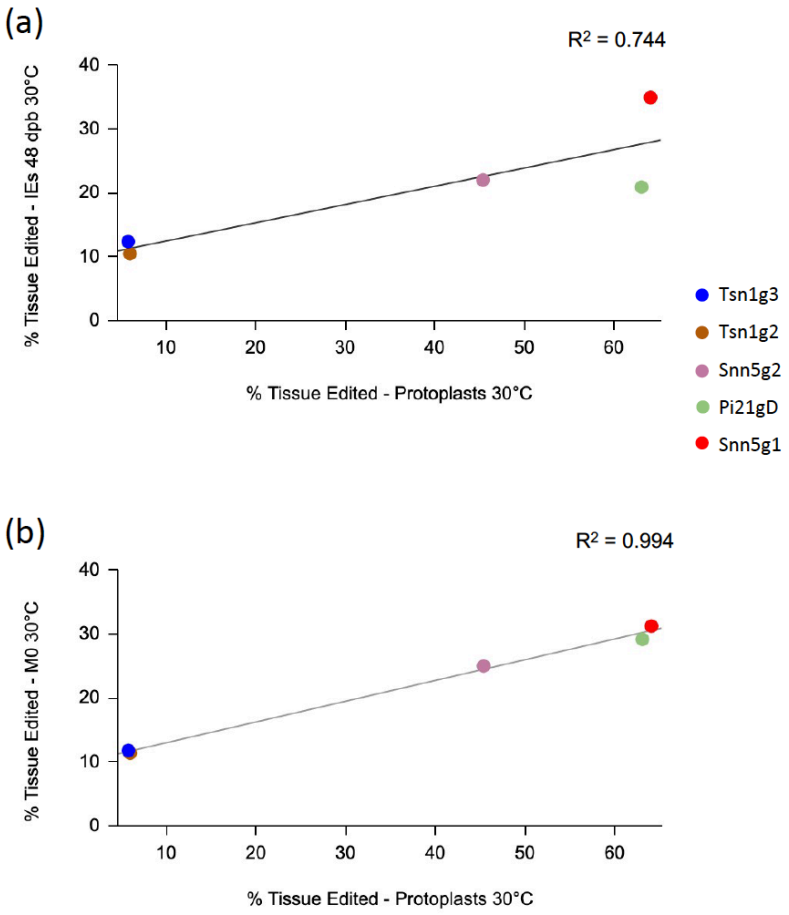


Figure 19. Correlation plot between targeted editing efficiency of gRNA-Cas9 RNPs in protoplasts and immature embryos (IEs) at **(a)** 48 dpb and in **(b)** M_0 plants treated at 30°C.

4.6 Cas9-RNP mediated knockout of *Parastagonospora nodorum* necrotrophic effector sensitivity genes

The wheat genes *Tsn1* and *Snn5* recognize necrotrophic effectors produced by *Parastagonospora nodorum*, and each exist as single copy genes on the B genome of allohexaploid wheat. In this study, 20 M_0 *Tsn1* edited plants were produced from 30 transfected embryos maintained for regeneration. Of those, 14 had heterozygous mutations and 6 had biallelic or homozygous mutations. Fully expanded secondary leaves of a subset of M_0 *Tsn1* edited plants, M_0 *Tsn1* WT plants, and Fielder grown from seed were infiltrated with SnToxA expressed in *Pichia*

pastoris. After 72 hours, M_0 heterozygotes, M_0 WT, and Fielder plants had necrotic lesions extending from the site of infiltration. Meanwhile, M_0 plants with biallelic or homozygous mutations exhibited no necrosis (Fig. 20).

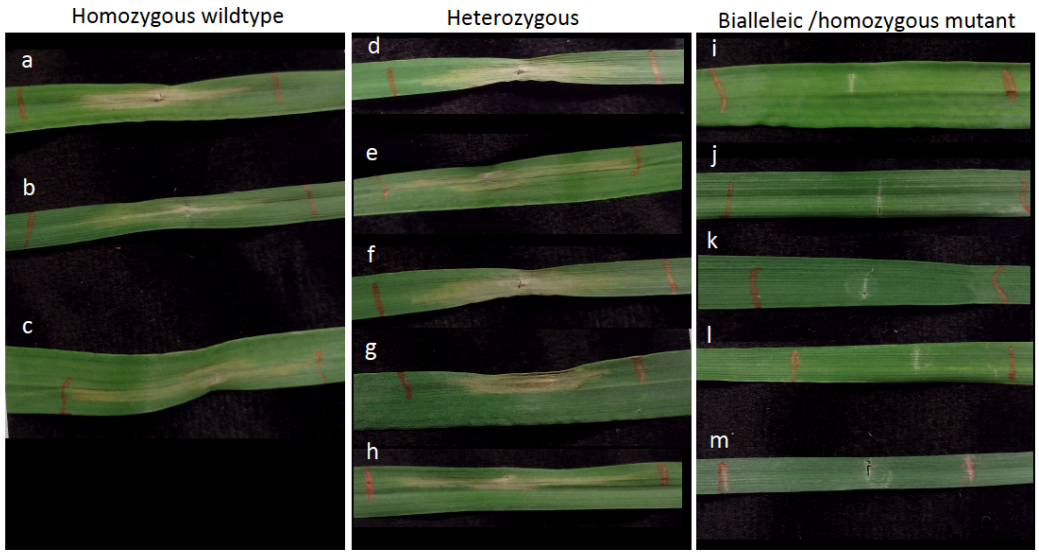


Figure 20. SnToxA assay in *Tsn1* targeted M_0 regenerants. (a) Fielder control grown from seed. (b-m) independent M_0 regenerants with (b, c) homozygous wildtype; (d-h) heterozygous (d) -2; (e) -5; (f) -31; (g) -1; (h) +1; and (i-m) biallelic or homozygous mutant (i) -2, -5; (j) -2, -2, (k) -1, -1; (l) -2, -2; (m) -1, -1 genotypes. Mutation notation is as follows: a positive number ,+, indicates the number of bases inserted, a negative number, -, indicates the number of bases deleted.

Similarly, a total of 24 M_0 *Snn5* edited plants were produced from 30 transfected embryos maintained for regeneration. Of those, 14 had heterozygous mutations and ten had biallelic or homozygous mutations. Fully expanded secondary leaves of a subset of M_0 *Snn5* edited plants, M_0 *Snn5* WT plants, and Fielder grown from seed were infiltrated with SnTox5 containing culture filtrates. After 72 hours, M_0 heterozygotes with in-frame deletions, M_0 WT, and Fielder plants exhibited necrotic lesions. Results for M_0 heterozygotes, however, displayed a mixture of phenotypes ranging from sensitive to insensitive. Two heterozygous plants with an in-frame deletion on one allele appeared insensitive to SnTox5. Notably, all plants with biallelic or homozygous mutations leading to premature termination were insensitive to SnTox5 (Fig. 21).

These results demonstrate that loss-of-function mutations can be introduced to both copies of a gene within the M_0 generation, leading to insensitivity to agronomically relevant necrotrophic fungal effectors. M_0 heterozygotes and biallelic plants can be self-fertilized to establish lines with homozygous deleterious mutations in the susceptibility genes. The biolistic method with 30°C or 37°C heat treatment is highly efficient, and edited plants can be identified from a small number of regenerants without the use of selection in tissue culture.

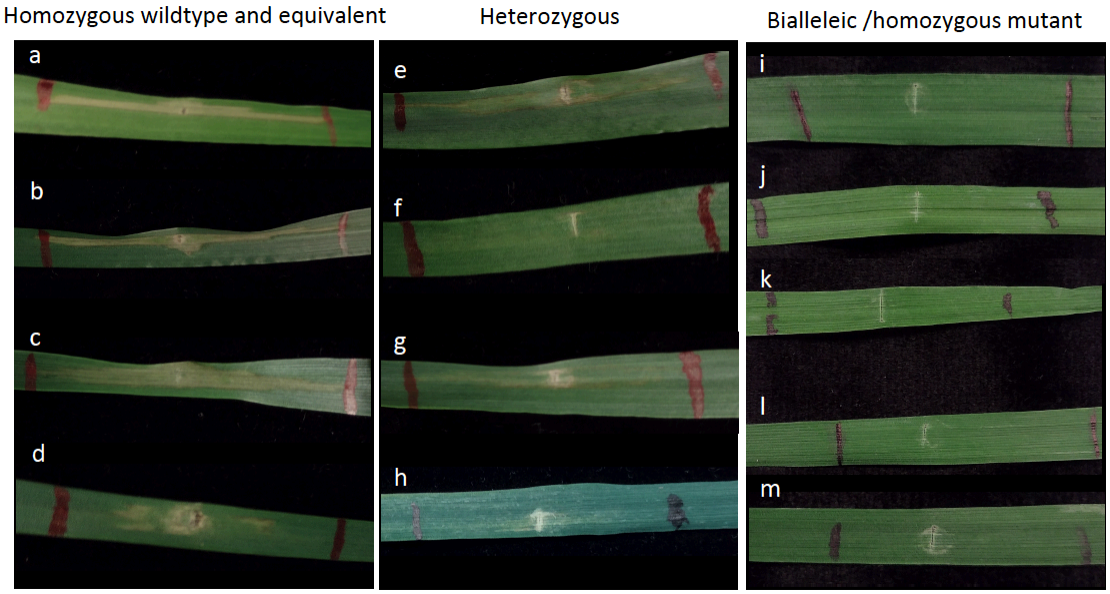


Figure 21. SnTox5 assay in *Snn5* targeted M_0 regenerants. (a) Fielder control grown from seed. (b-m) independent M_0 regenerants with (b) homozygous wildtype; (c-d) heterozygous in-frame mutant: (c) -3; (d) -6; (e-h) heterozygous mutant: (e) -5; (f) +20; (g) +2-1; (h) -4; (i-m) biallelic or homozygous mutant: (i) -11, -4; (j) -8, -2; (k) -10, -10; (l) +1, -2; (m) -5, -1 genotypes. Mutation notation is as follows: a positive number,+, indicates the number of bases inserted, a negative number, -, indicates the number of bases deleted.

4.7 Discussion

CRISPR-based RNPs have been used for editing in various plant species and tissue types [90]. In this work, we improve upon DNA-free Cas9-RNP technology for genome editing in wheat. We establish heat treatment as a parameter to increase the rate of editing *in vivo*, show that particle bombardment-based editing is sustained over more than 14 days, and demonstrate that results from protoplast assays can be utilized as a proxy for predicting editing rates in regenerable tissue and as a tool to rank gRNA efficacy. By delivering gene editing reagents as protein-RNA complexes, several complications associated with *Agrobacterium tumefaciens,* and biolistic DNA vector delivery are avoided.

Cas9 from *Streptococcus pyogenes*, a bacterium that grows optimally at 37°C [91], has been shown to exhibit increased cleavage activity at 37° compared to 22°C *in vitro* [68]. Plant protoplast and IE transfections and regeneration are typically performed at ambient temperatures (25°C and 26°C respectively). Although modulation of temperature has not been previously performed in protoplast gene editing experiments, an increase in temperature for DNA-based plant gene editing studies have resulted in higher targeted mutation frequencies [68,92]. The application of temperature treatment to increase Cas9-RNP mediated editing efficiency in any plant tissue system has not previously been demonstrated. Here, we found that 16 hours of exposure of Cas9-RNP transfected protoplasts to 30°C markedly increased indel formation at the Cas9 cut site (Fig. 15). Similarly, 16 hours of exposure of Cas9-RNP bombarded IEs to 30°C or 37°C resulted in increased targeted indel formation. In IEs assayed at 48 dpb we achieved editing rates of 10.4-34.9% with 30°C treatment, 6.63-24.39% with 37°C treatment, and just 3.36-14.25% with standard 26°C incubation

(Fig. 16b). Interestingly, the benefit of increased temperature treatment was consistent between the two target tissues and across the five different target sites tested. In our work, there were no discernable defects in regenerability for IEs treated at a higher temperature compared to the standard 26°C. We detected no positive or negative correlation between temperature treatment and the number of M_0 plants recovered.

Two reports have described the biolistic delivery of Cas9-RNPs into wheat and maize embryo cells in the absence of DNA and selection [22,24]. Both achieved moderate targeted mutagenesis frequencies in the regenerated plants. We noted that the studies each assayed for editing efficiency in the IEs 2 dpb and universally achieved <1% targeted editing. In contrast, the editing efficiencies in regenerated plant tissue were substantially higher, ranging from 1.3-4.7% [24] and 2.4-9.7% [22]. To investigate this discrepancy between timepoints, we monitored editing efficiency at 14 dpb, 48 dpb, and in the M_0 regenerants in our study. Irrespective of temperature treatment or gRNA sequence, editing frequencies at 48 dpb were considerably higher than at 14 dpb (Figure 16b). Percentage of tissue edited in the M_0 plant pool was comparable to that at 48 dpb. The observed difference in editing efficiency between earlier timepoints and regenerated M_0 plants was consistent with previous reports [22,24].

In mammalian cells, Cas9 was shown to be undetectable 48-72 hours after Cas9-RNP transfection by nucleofection [25]. For this reason, it has been thought that enzymatic degradation of Cas9-RNPs *in vivo* is rapid and that editing must occur within the first few days of transfection. In the present study, if Cas9-RNPs were fully degraded from the tissue prior to the 14 dpb timepoint, all gene editing would have had to occur before 14 dpb. Consequently, approximately the same number of unique alleles would have been expected to be detected at both 14 dpb and 48 dpb if proliferation of edited and unedited cells occurs at the same rate. On the contrary, consistently higher rates of mutagenesis as well as a greater number of unique alleles at the later timepoints were observed at 48 dpb (Figure 16b, Figure 17a, Figure 18), suggesting that Cas9 may somehow be stabilized for at least 14 days and gradually released within the wheat IEs after biolistic delivery for sustained editing over time. As further evidence in support of this hypothesis, Cas9 protein was detected in 10-embryo tissue samples taken 2, 7, and 14 dpb (Figure 17b). Taken together, these results indicate that Cas9 is maintained in tissue at least 14 dpb and facilitates sustained and gradual editing of tissue over time when delivered as Cas9-RNP via gold particle bombardment. Further biochemical exploration is necessary to understand the mechanism of this Cas9 stabilization and persistent editing.

Numerous plant protoplast systems have been used for targeted mutagenesis using Cas9-RNPs [42,51,86-89,93]. Although the method is useful for producing Cas9-RNP edited plants for protoplasts that are amenable to regeneration, most crop plants cannot easily be regenerated in this manner. Though wheat protoplasts are recalcitrant to regeneration through existing methodology, protoplasts in the current study prove to be a beneficial screening system. Cas9-RNP mediated editing rates in protoplasts correlated linearly with editing rates in IEs. Because biolistic Cas9-RNP transfection of IEs requires significant time, energy, resources, and commitment, a means for rational selection of gRNA sequences for optimal editing efficiency is preferred. It is noteworthy that there were major differences in mutation rates for the 5 gRNAs used in this study. Unfortunately, existing predictive software to select gRNAs often do not translate upon experimentation. Therefore, when attempting to select the best gRNA to produce the highest rate of stable editing in regenerable IEs, transient protoplast assays can serve as a rapid pipeline to rank gRNAs and forecast editing rates in Cas9-RNP bombarded regenerable tissue.

The calculation of editing efficiency in M_0 regenerants has the potential to be confusing. To be explicit in our analysis, we present editing rates of regenerants in two ways. The percentage of total edited alleles in the M_0 regenerant pool is indicated as "% Tissue edited", while "% Plants edited" is the percentage of total edited plants among all the M_0 plants (Fig 16b, Fig. 19, Table 6). The former is meant to compare overall editing efficiency more fairly across tissue types and timepoints, taking biallelism, homozygosity, and heterozygosity of regenerated plants into consideration. The latter value is more relevant for evaluating the method's ability to produce individual plants with gene edits.

The gene *Pi21* was first characterized in rice (*Oryza sativa*) as a negative regulator of resistance for blast disease [15]. We identified putative orthologs in wheat that consisted of three homoeologous genes. In Chapter 3, we discussed findings that indicated that despite our confidence in orthology, homozygous triple knockout of wheat *Pi21* homoeologs did not yield any disease resistance phenotypes. Therefore, wheat *Pi21* was selected simply as a target to demonstrate the utility and feasibility of DNA-free Cas9-RNP gene editing method in a gene present in all three diploid subgenomes (AABBDD). Pi21gD was designed to simultaneously target all six alleles. Despite the genetic complexity, we were able to regenerate plants with biallelic or homozygous mutations across all three subgenomes for a full variety of genotypes including two with homozygous triple mutant knockouts within the M_0 generation.

The wheat genes *Tsn1* and *Snn5* recognize the *Parastagonospora nodorum* pathogenic effectors SnToxA and SnTox5, respectively [94,95]. *Tsn1* is a gene with resistance gene-like features including protein kinase, nucleotide binding, and leucine-rich repeats, and the ToxA necrotrophic effector is produced by at least three economically important fungal pathogens of wheat [96]. *Snn5* belongs to a different class and contains protein kinase and major sperm protein domains (details regarding the cloning and characterization of *Snn5* will be published in the future; K.L.D. Running and J.D. Faris, personal communication), but like *Tsn1*, it functions as a target for a necrotrophic effector leading to disease susceptibility [95]. Therefore, *Tsn1* and *Snn5* are practical targets for disruption via DNA-free gene editing. Using DNA-free biolistic delivery of Cas9-RNPs, we successfully generated plants with heterozygous, biallelic, and homozygous mutations within the M_0 generation from a mere ten IEs per treatment. Biallelic and homozygous mutants of *Tsn1* and *Snn5* were demonstrated to be insensitive to SnToxA and SnTox5 respectively. Due to the high rate of editing, particularly using Snn5g1 and Snn5g2 with 30°C and 37°C heat treatment, screening of M_0 plants for edits was fully feasible. Contrary to previous reports, a selection scheme can reasonably be foregone with Cas9-RNP mediated editing so long as gRNAs are pre-tested in protoplasts and deemed to be highly effective.

In summary, heat treatment enhancement of Cas9-RNP mediated wheat editing combined with a protoplast-based approach to select optimal gRNAs, and findings that editing is sustained for more than 2 weeks advances this DNA and selection-free gene editing approach in crops. Given the persistence of Cas9 in bombarded tissue, additional work with increased length or punctuated exposure to heat, beyond 16 hours, throughout callus induction may further augment the benefit of heat treatment. The success of this method in targeting single loci warrants exploration of furthering the technique to multiplexing. In addition to knocking out genes, editing via Cas9-RNPs can conceivably be applied to generating allelic series by targeting non-coding genomic regions such as promoters [97]. The presented advancement to this technology can be applied to numerous crops that are amenable to particle bombardment and encourages the establishment of tissue culture and regeneration protocols in crop species that are vegetatively propagated.

4.8 Materials and Methods

Plant material

The allohexaploid wheat (*Triticum aestivum* L., 2*n* = 6*x* = 42, AABBDD genomes) cultivar Fielder was used for this study.

Cas9-gRNA RNP assembly

Cas9 protein with a C-terminal double nuclear-localization tag (QB3 Macrolab, University of California, Berkeley) and sgRNAs with modifications of 2'-O-Methyl at 3 first and last bases, and 3' phosphorothioate bonds between first 3 and last 2 bases (Synthego, Menlo Park, CA) were complexed *in vitro* to form Cas9-gRNA RNPs.

For each protoplast transfection, a 25 µl reaction was assembled. Thoroughly mixed were 10 µg sgRNA, 2.5 µl 10X NEBuffer 3.1 (New England Biolabs, Ipswich, MA), and nuclease-free water. Then, in a drop-wise manner, 10 µg Cas9 was added slowly with constant mixing, followed by 20 min incubation at 37°C.

For each IE biolistic transfection, a 40 ul reaction was assembled. Thoroughly mixed were 6.4 µg sgRNA, 4 µl 10X NEBuffer 3.1, and nuclease-free water. Then, in a drop-wise manner, 12.8 µg Cas9 was added slowly with constant mixing, followed by 20 min incubation at 37°C.

The resultant RNP mixtures were stored on ice until transfection. The gRNA sequences are as follows:

gRNA ID	Target Site Sequence
Pi21gD	ACAACAGGGTGATCGTCCGT
Tsn1g2	GGAACTGTCTACTAATATAT
Tsn1g3	ACCATAAAGGGGATTTGTGA
Snn5g1	ATACAATGGAGAACTAGTTA
Snn5g2	CTTGCAAGGACTTGATGATA

Protoplast isolation and transfection

Partially etiolated seedlings were used as donor tissue for protoplast isolation. Seeds were surface sterilized in 20% (v/v) bleach and rinsed in sterile water. Seedlings were grown under sterile conditions on wet filter paper in the dark for 12-14 days at 25°C with exposure to ambient light for 6 hours every 5 days. Wheat protoplasts were isolated from the donor tissue using a previously described method [11]. For each transfection 25 µl of Cas9-gRNA RNP mixture, as defined above, were added to 5 X 10^5 protoplasts. PEG-meditated transfection was performed as described in the literature [11]. Protoplasts were harvested 24 hours post-transfection for analysis.

Gold particle preparation for bombardment

Cas9-RNPs were precipitated onto 0.6 µm gold particles (#1652262, Bio-Rad, Hercules, CA) using the cationic lipid polymer TransIT-2020 (Mirus, Madison, WI) as previously described [22], with modifications. Briefly, for each 30-IE transfection, 40 µl Cas9-RNP mixture, as described above, was mixed gently with 20 µl sterile gold particles (10 µg μl^{-1} water suspension) and 1 µl TransIT-2020 and incubated on ice for 20 min. The Cas9-RNP coated gold particles were pelleted in a mini microcentrifuge at 2,000*g* for 30 s. The supernatant was removed, and the gold particles were resuspended in 20 µl of sterile water by brief sonication. The coated gold particles were immediately applied to 2 macrocarriers (10 µl each) by spotting numerous small drops and allowed

to air dry in a laminar flow hood. For a single transfection, each 30-IE set was bombarded twice using the 2 prepared macrocarriers.

Immature embryo bombardment and regeneration

Plants were grown at 24°C, 16-hour days and 15°C, 8-hour nights under light intensity of 130 µmol $m^{-2}s^{-1}$. Immature seeds containing IEs, sized 1.7-2.2 mm were harvested from wheat spikes 10-13 days after flowering, surface sterilized in 20% (v/v) bleach with one drop of Tween 20 and triple rinsed with sterile water, followed by extraction of the IEs. The IEs were placed on DBC3 media [66], scutellum side up and incubated overnight at 26°C prior to biolistic transfection. Four hours prior to bombardment, IEs were placed on 55 mm filter paper in the center of DBC3 osmoticum media containing 0.2 M mannitol and 0.2 M sorbitol [49]. Using two prepared microcarriers holding Cas9-RNP coated gold microparticles, IEs were shot twice using the PDS-100/He gene gun (Bio-Rad, Hercules, CA) with rupture pressure of 1100 psi. The bombarded IEs were transferred from the filter paper directly to the media below and incubated at 26°C, 30°C, or 37°C for 16 hours. IEs were transferred to standard DBC3 media in dim light (10 µmol m^{-2} s^{-1}) at 26°C for 9 weeks with subculturing as needed. Callus tissue originating from each IE was transferred to DBC6 media for regeneration [70]. Resultant plantlets were transferred to rooting media and incubated in high light (90 µmol m^{-2} s^{-1}) at 26°C and grown to 4-6 inches before being transplanted to soil.

Amplicon next generation sequencing analysis

To determine mutation rates by amplicon sequencing, PCR was performed with target-specific primers (Table 7), amplifying approximately 225 bp around the cut site using Phusion High Fidelity (New England Biolabs, Ipswich, MA) polymerase. Primers contained a 5′-stub compatible with Illumina NGS library preparation. PCR products were ligated to Illumina TruSeq adaptors and purified. Libraries were prepared using a NEBNext kit (Illumina, San Diego, CA) according to the manufacturer's guidelines. Samples were deep sequenced on an Illumina iSeq at 200 bp paired-end reads to a depth of approximately 10,000 reads per sample. Cortado (https://github.com/staciawyman/cortado) was used to analyze editing outcomes. Briefly, reads were adapter trimmed then merged using overlap to single reads. These joined reads were then aligned to the target reference sequence. Editing rates are calculated by counting any reads with an insertion or deletion overlapping the cut site or occurring within a 3 bp window on either side of the cut site. SNPs occurring within the window around the cut site are not counted. Total edited reads are then divided by the total number of aligned reads to derive percent edited.

Table 7: Primers used to amplify the target region for amplicon next generation sequencing. Nucleotides shown in capital letters are the 5′-stub compatible with Illumina NGS library preparation.

Amplicon target	F Primer	R Primer
Pi21gD	GCTCTTCCGATCTagttcttcttacgtaagattgatcata	GCTCTTCCGATCTcaggccttgcaccagatctt
Tsn1g2 / Tsn1g3	GCTCTTCCGATCTggaaactgatttctc	GCTCTTCCGATCTcaaaatccgccagtt
Snn5g1	GCTCTTCCGATCTtgacagtgaattccgtaacc	GCTCTTCCGATCTtagtaatgtggagcaccttc
Snn5g2	GCTCTTCCGATCTgctgactacaaacagattgtcc	GCTCTTCCGATCTtaactatttggtagcagtagcc

Western blot

Total plant tissue originating from 10 IEs at different timepoints were frozen in LN2, ground to a fine powder by mortar and pestle, and resuspended in 200 µl 2x Laemmli Sample Buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol. Samples were boiled for 5 min, and the total soluble protein extracts (25 µl or 40 µl per well) were separated on 4-20% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad, Hercules, CA) and subsequently transferred to a 0.45 µm nitrocellulose membrane (GVS, Sanford, ME). For detection of Cas9 protein, anti-CRISPR/Cas9 C-terminal mouse monoclonal antibody (SAB4200751; Sigma-Aldrich, St. Louis, MO) and ProSignal Dura ECL Reagent (Genesee Scientific, San Diego, CA) were used. PageRuler Plus Prestained Protein Ladder (10–250 kDa, Thermo Fisher, Waltham, MA) was used as a molecular weight marker, and Cas9 protein with a C-terminal double nuclear-localization tag (QB3 Macrolab, University of California, Berkeley) was used as a positive control.

Production of SnToxA

SnToxA was expressed in the *Pichia pastoris* yeast strain X33 [98] and cultured in yeast peptone dextrose broth (10 g yeast extract, 20 g peptone, 100 ml 20% dextrose in 900 ml distilled water) for 48 hours at 30 °C. Culture filtrate was harvested and filtered through a 0.45 µm HVLP filter membrane (Merk Millipore Ltd., Cork, Ireland) and dialyzed overnight against water using 3.5 kDa molecular weight cut off Snake Skin dialysis tubing (Thermo Scientific, IL, USA). Dialyzed filtrate was loaded onto a HiPrep Sp XL 16/10 cation exchange column (GE Healthcare Piscataway, NJ). Unbound protein was washed off the column using a 20 mM sodium acetate (pH 5.0) buffer prior to a gradient elution of SnToxA using a buffer consisting of 300 mM sodium chloride and 20 mM sodium acetate (pH 5.0). Fractions that contained SnToxA were collected and frozen prior to lyophilizing to increase the concentration of SnToxA. Lyophilized samples were dissolved in a buffer consisting of 5 mM MOPS sodium salt (Alfa Aesar, MA, USA) and water, prior to infiltration into the plants.

Production of SnTox5

P. nodorum strain Sn79+Tox5-3, generated by transforming *SnTox5* in to the avirulent *P. nodorum* strain Sn79-1087 [95],was used to prepare the culture filtrates containing SnTox5 as previously described [99] with minor modifications. In brief, Sn79+Tox5-3 was grown on V8-potato dextrose agar medium till spores were released from pycnidia. The plates were flooded with 10 ml of sterile distilled water, and 500 µl of spore suspension was used to inoculate 60 ml of liquid Fries

medium (5 g ammonium tartrate, 1 g ammonium nitrate, 0.5 g magnesium sulfate, 1.3 g potassium phosphate [dibasic], 3.41 g potassium phosphate [monobasic], 30 g sucrose, 1 g yeast extract in 1000 ml of distilled water). Cultures were grown on an orbital shaker at 100 rpm for a week prior to two weeks of stationary growth under dark conditions at room temperature. Culture filtrates were filtered through a layer of Miracloth (EMD Millipore Corp, MA, USA) and were concentrated 5-fold using Amicon Ultracel – 3K centrifugal filters (Merk Millipore Ltd., Cork, Ireland). Culture filtrates were diluted in a 1:1 ratio with sterile water prior to infiltration into the plants.

Necrotrophic effector infiltrations

Infiltrations with SnToxA and SnTox5 containing culture filtrates were conducted as previously described [99]. Three infiltrations were performed per plant, and sensitivity was evaluated on a binary scale at 3 days post infiltration.

CHAPTER 5. Concluding remarks

Plant biotechnology and agriculture are being revolutionized by the ability to utilize CRISPR-Cas9 to precisely modify complex genomes via gene editing. I have described here a set of studies that advance enabling technology and genome editing in monocot crops for disease resistance and sustainability. As research continues, I believe the field will greatly benefit from further expansion of the plant gene editing toolkit.

A large dataset describing Cas9-mediated gene editing efficiency and specific mutational outcomes using many gRNA sequences *in planta* to train predictive models would be of high value to the community. Currently, computational gRNA predictive software is trained on gene editing data from mammalian cells. Quite often, we find that these prediction tools do not correlate with *in vivo* editing results in plants. Because of this, protoplast assays, like those I described in this body of work, are well suited for quickly ranking Cas9-RNA efficacy in the absence of such software. Protoplasts assays themselves can additionally be used to amass the necessary data to train new models so that gRNA efficacy predictive software can be made a reliable tool for the future of plant gene editing.

By generating homozygous triple mutant knockout lines without the need for additional crossing in allohexaploid wheat using both plasmid and RNP Cas9 gene editing systems, we have demonstrated that even the most complex genomes can be edited in just a single round of transfections. While wheat has moderately short generation times and can feasibly be crossed to other lines, plants with long generation times and vegetatively propagated plants will rely on gene editing pipelines that can create full homozygous mutations in a single round. More importantly, they will rely on DNA-free gene editing that does not require crossing to eliminate transgenes. Our improvements to Cas9-RNP selection and DNA-free methods for gene editing in wheat provides a platform to be translated for use in these plants. In addition to this, work exploring multiplexing and alternative DNA-free gene editing techniques will be important.

The work described in this dissertation focused on editing in the form of targeted loss of function mutations. While many beneficial traits can be obtained in this manner, the next step for gene editing in crops will be precise integration of exogenous advantageous alleles. Although non-homologous end joining has been described as the dominant double-stranded break repair pathway in plants, ongoing research is working to strengthen and harness homology directed repair for precise genome edits from single base pair replacements to insertions of long fragments or genes. The establishment of highly efficient gene editing in plants by homology directed repair will be a significant breakthrough for crop bioengineering. Additionally, as more CRISPR endonucleases beyond Cas9 are discovered and controlled for use in biotechnology, editing parameters will become even less limited. With these discoveries will come the ability to edit regions unconstrained by specific protospacer associated motifs, improvements in precision, and new types of single and double stranded breaks in both DNA and RNA. The potential for crop genome editing is immense, and with the technologies already in hand as well as those to come, we can work to address and improve agricultural issues of food security and climate change.

REFERENCES

1. Desta, Z. A. & Ortiz, R. Genomic selection: genome-wide prediction in plant improvement. *Trends Plant Sci* **19,** 592–601 (2014).
2. Collard, B. C. Y. & Mackill, D. J. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philos Trans R Soc Lond B Biol Sci* **363,** 557–572 (2008).
3. Lidder, P. & Sonnino, A. Biotechnologies for the management of genetic resources for food and agriculture. *Adv Genet* **78,** 1–167 (2012).
4. Wolter, F., Schindele, P. & Puchta, H. Plant breeding at the speed of light: the power of CRISPR/Cas to generate directed genetic diversity at multiple sites. *BMC Plant Biology* **19,** 176–8 (2019).
5. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337,** 816–821 (2012).
6. Li, J., Li, Y. & Ma, L. CRISPR/Cas9-Based Genome Editing and its Applications for Functional Genomic Analyses in Plants. *Small Methods* **3,** 1800473 (2019).
7. Symington, L. S. & Gautier, J. Double-strand break end resection and repair pathway choice. *Annu Rev Genet* **45,** 247–271 (2011).
8. Li, Z. & Murai, N. Efficient plant regeneration from rice protoplasts in general medium. *Plant Cell Rep.* **9,** 216–220 (1990).
9. Utomo, H. S., Croughan, S. S. & Croughan, T. P. Suspension and protoplast culture of U.S. rice cultivars. *Plant Cell Rep.* **15,** 34–37 (1995).
10. He, F., Chen, S., Ning, Y. & Wang, G.-L. Rice (Oryza sativa) Protoplast Isolation and Its Application for Transient Expression Analysis. *Current Protocols in Plant Biology* **1,** 373–383 (2016).
11. Shan, Q., Wang, Y., Li, J. & Gao, C. Genome editing in rice and wheat using the CRISPR/Cas system. *Nature Protocols* **9,** 2395–2410 (2014).
12. Page, M. T., Parry, M. A. J. & Carmo Silva, E. A high-throughput transient expression system for rice. *Plant Cell Environ* **42,** 2057–2064 (2019).
13. Kato, H. *et al.* Pathogenicity, Mating Ability and DNA Restriction Fragment Length Polymorphisms of Pyricularia Populations Isolated from Gramineae, Bambusideae and Zingiberaceae Plants. *J Gen Plant Pathol* **66,** 30–47 (2000).
14. Igarashi, S., Utiamada, C. M., Igarashi, L. C., Bras, A. K. P. S. F.1986. *Pyricularia in wheat. 1. Occurrence of Pyricularia sp*.
15. Fukuoka, S. *et al.* Loss of function of a proline-containing protein confers durable disease resistance in rice. *Science* **325,** 998–1001 (2009).
16. Zhou, X. *et al.* Loss of function of a rice TPR-domain RNA-binding protein confers broad-spectrum disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* **115,** 3174–3179 (2018).
17. Wang, Y. *et al.* Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology* **32,** 947–951 (2014).
18. Zhang, Y. *et al.* Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. *Plant J.* **91,** 714–724 (2017).
19. Sánchez-León, S. *et al.* Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnology Journal* **16,** 902–910 (2018).

20. Howells, R. M., Craze, M., Bowden, S. & Wallington, E. J. Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. *BMC Plant Biology* **18,** 215–11 (2018).
21. Zhang, S. *et al.* Highly Efficient and Heritable Targeted Mutagenesis in Wheat via the Agrobacterium tumefaciens-Mediated CRISPR/Cas9 System. *Int J Mol Sci* **20,** 4257 (2019).
22. Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K. & Mark Cigan, A. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat Commun* **7,** 13274 (2016).
23. Banakar, R. *et al.* High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice. *Scientific Reports* **9,** (2019).
24. Liang, Z. *et al.* Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat Commun* **8,** 14261 (2017).
25. Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J.-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **24,** 1012–1019 (2014).
26. Birla, D. S. *et al.* Progress and challenges in improving the nutritional quality of rice (Oryza sativa L.). *Critical Reviews in Food Science and Nutrition* **57,** 2455–2481 (2017).
27. Jackson, S. A. Rice: The First Crop Genome. *Rice* 1–3 (2016). doi:10.1186/s12284-016-0087-4
28. Romero, F. M. & Gatica-Arias, A. CRISPR/Cas9: Development and Application in Rice Breeding. *Elsevier India Pvt Ltd.* **26,** 265–281 (2019).
29. Sheen, J. Signal Transduction in Maize and Arabidopsis Mesophyll Protoplasts. *Plant Physiol.* **127,** 1466–1475 (2001).
30. Su, P.-H., Yu, S.-M. & Chen, C.-S. Spatial and temporal expression of a rice prolamin gene RP5 promoter in transgenic tobacco and rice. *Journal of Plant Physiology* **158,** 247–254 (2001).
31. Ni, J. *et al.* Heterologous Expression and Functional Analysis of Rice GLUTAMATE RECEPTOR- LIKE Family Indicates its Role in Glutamate Triggered Calcium Flux in Rice Roots. *Rice* 1–14 (2016). doi:10.1186/s12284-016-0081-x
32. Hiei, Y. & Komari, T. Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature Protocols* **3,** 824–834 (2008).
33. Andrieu, A. *et al.* An in planta, Agrobacterium-mediated transient gene expression method for inducing gene silencing in rice (Oryza sativa L.) leaves. *Rice* **5,** 23–12 (2012).
34. Christou, P. Genetic transformation of crop plants using microprojectile bombardment. *Plant J.* 1–7 (1992).
35. Kirienko, D. R., Luo, A. & Sylvester, A. W. Reliable Transient Transformation of Intact Maize Leaf Cells for Functional Genomics and Experimental Study. *Plant Physiol.* **159,** 1309–1318 (2012).
36. Prelich, G. Gene Overexpression: Uses, Mechanisms, and Interpretation. *Genetics* **190,** 841–854 (2012).
37. Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S. & Venkataraman, G. CRISPR for Crop Improvement: An Update Review. *Front. Plant Sci.* **9,** 117–17 (2018).
38. Romero, F. M. & Gatica-Arias, A. CRISPR/Cas9: Development and Application in Rice Breeding. *Elsevier India Pvt Ltd.* **26,** 265–281 (2019).
39. Wu, F.-H. *et al.* Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method. *Plant Methods* **5,** 16–10 (2009).

40. Yao, X. *et al.* Preparation and applications of guard cell protoplasts from the leaf epidermis of Solanum lycopersicum. *Plant Methods* 1–9 (2018). doi:10.1186/s13007-018-0294-7
41. Gao, L. *et al.* An efficient system composed of maize protoplast transfection and HPLC–MS for studying the biosynthesis and regulation of maize benzoxazinoids. *Plant Methods* 1–13 (2019). doi:10.1186/s13007-019-0529-2
42. Shan, X. *et al.* Efficient isolation of protoplasts from freesia callus and its application in transient expression assays. *Plant Cell, Tissue and Organ Culture (PCTOC)* **138,** 529–541 (2019).
43. Priyadarshani, S. V. G. N. *et al.* Simple protoplast isolation system for gene expression and protein interaction studies in pineapple (Ananas comosus L.). *Plant Methods* 1–12 (2018). doi:10.1186/s13007-018-0365-9
44. Toriyama, K. & Hinata, K. Cell Suspension and Protoplast Culture in Rice. *Plant Science* 1–5
45. Thompson, J. A., Abdullah, R. & Cocking, E. C. Protoplast Culture of Rice (Oryza Sative L.) Using Media Solidified with Agarose. *Plant Science* 123–133
46. Shan, Q., Wang, Y., Li, J. & Gao, C. Genome editing in rice and wheat using the CRISPR/Cas system. *Nature Protocols* **9,** 2395–2410 (2014).
47. Page, M. T., Parry, M. A. J. & Carmo Silva, E. A high-throughput transient expression system for rice. *Plant Cell Environ* **42,** 2057–2064 (2019).
48. Nanjareddy, K., Arthikala, M.-K., Blanco, L., Arellano, E. S. & Lara, M. Protoplast isolation, transient transformation of leaf mesophyll protoplasts and improved Agrobacterium-mediated leaf disc infiltration of Phaseolus vulgaris: tools for rapid gene expression analysis. *BMC Biotechnology* 1–14 (2016). doi:10.1186/s12896-016-0283-8
49. Cho, M.-J., Ha, C. D. & Lemaux, P. G. Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues. *Plant Cell Rep.* **19,** 1084–1089 (2000).
50. Mathur, J. & Koncz, C. in *Arabidopsis Protocols* (eds. Martinez-Zapater, J. M. & Salinas, J.) 267–276 (Humana Press, 1998).
51. Malnoy, M. *et al.* DNA-Free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci.* **7,** 1473 (2016).
52. Uauy, C., Wulff, B. B. H. & Dubcovsky, J. Combining Traditional Mutagenesis with New High-Throughput Sequencing and Genome Editing to Reveal Hidden Variation in Polyploid Wheat. *Annu Rev Genet* **51,** 435–454 (2017).
53. Nadolska-Orczyk, A., Rajchel, I. K., Orczyk, W. & Gasparis, S. Major genes determining yield-related traits in wheat and barley. *Theor Appl Genet* **130,** 1081–1098 (2017).
54. Feldman, M. & Levy, A. A. Genome evolution due to allopolyploidization in wheat. *Genetics* **192,** 763–774 (2012).
55. 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* **39,** 70–82 (2006).
56. Puchta, H. The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J. Exp. Bot.* **56,** 1–14 (2005).
57. Fernandez, J. & Orth, K. Rise of a Cereal Killer: The Biology of Magnaporthe oryzae Biotrophic Growth. *Trends Microbiol.* (2018). doi:10.1016/j.tim.2017.12.007

58. Prabhu, A. S., Filippi, M. C. & Castro, N. Pathogenic variation among isolates of Pyricularia oryzae affecting rice, wheat, and grasses in Brazil. *International Journal of Pest Management* (2008). doi:10.1080/09670879209371729
59. Cruz, C. D. & Valent, B. *Wheat blast disease: danger on the move. Tropical Plant Pathology, 42, 210-222*. (2017).
60. Yoshida, K. *et al.* Host specialization of the blast fungus Magnaporthe oryzae is associated with dynamic gain and loss of genes linked to transposable elements. *BMC Genomics* **17,** 1–18 (2016).
61. Islam, M. T. *et al.* Wheat blast: a new threat to food security. *Phytopathol Res* **2,** 1–13 (2020).
62. Anh, V. L. *et al.* Rmg8, a New Gene for Resistance to Triticum Isolates of Pyricularia oryzae in Hexaploid Wheat. *Phytopathology* (2015). doi:10.1094/PHYTO-02-15-0034-R
63. Wang, S. *et al.* A New Resistance Gene in Combination with Rmg8 Confers Strong Resistance Against Triticum Isolates of Pyricularia oryzae in a Common Wheat Landrace. *Phytopathology* (2018). doi:10.1094/PHYTO-12-17-0400-R
64. Inoue, Y., Vy, T. T. P., Tani, D. & Tosa, Y. Suppression of wheat blast resistance by an effector of Pyricularia oryzae is counteracted by a host specificity resistance gene in wheat. *New Phytol* **229,** 488–500 (2021).
65. Guo, H. *et al.* Identification of Rice Blast Loss-of-Function Mutant Alleles in the Wheat Genome as a New Strategy for Wheat Blast Resistance Breeding. *Frontiers in Genetics* **12,** (2021).
66. Cho, M.-J., Jiang, W. & Lemaux, P. G. Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. *Plant Science* **138,** 229–244 (1998).
67. Peng, Z. *et al.* Long read and single molecule DNA sequencing simplifies genome assembly and TAL effector gene analysis of Xanthomonas translucens. *BMC Genomics* **17,** 21–19 (2016).
68. LeBlanc, C. *et al.* Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. *Plant J.* **93,** 377–386 (2018).
69. Milner, M. J., Craze, M., Hope, M. S. & Wallington, E. J. Turning Up the Temperature on CRISPR: Increased Temperature Can Improve the Editing Efficiency of Wheat Using CRISPR/Cas9. *Front. Plant Sci.* **11,** (2020).
70. Cho, M.-J., Banh, J., Yu, M., Kwan, J. & Jones, T. J. Improvement of Agrobacterium-mediated transformation frequency in multiple modern elite commercial maize (Zea mays L.) inbreds by media modifications. *Plant Cell, Tissue and Organ Culture (PCTOC)* **121,** 519–529 (2015).
71. Cruppe, G. *et al.* Novel Sources of Wheat Head Blast Resistance in Modern Breeding Lines and Wheat Wild Relatives. *Plant Dis* **104,** 35–43 (2020).
72. Valent, B., Farrall, L. & Chumley, F. G. Magnaporthe grisea genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* **127,** 87–101 (1991).
73. Li, J.-F. *et al.* Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nature Biotechnology* **31,** 688–691 (2013).
74. Wang, S. *et al.* Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. *Plant Cell Rep.* **34,** 1473–1476 (2015).

75. Butler, N. M., Atkins, P. A., Voytas, D. F. & Douches, D. S. Generation and inheritance of targeted mutations in potato (Solanum tuberosum L.) using the CRISPR/Cas system. *PLoS ONE* **10,** e0144591 (2015).
76. Brooks, C., Nekrasov, V., Lippman, Z. B. & Van Eck, J. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol.* **166,** 1292–1297 (2014).
77. Lor, V. S., Starker, C. G., Voytas, D. F., Weiss, D. & Olszewski, N. E. Targeted mutagenesis of the tomato PROCERA gene using transcription activator-like effector nucleases. *Plant Physiol.* **166,** 1288–1291 (2014).
78. Jacobs, T. B., LaFayette, P. R., Schmitz, R. J. & Parrott, W. A. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology* **15,** 16–10 (2015).
79. Svitashev, S. *et al.* Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize ssing Cas9 and guide RNA. *Plant Physiol.* **169,** 931–945 (2015).
80. Char, S. N. *et al.* An Agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. *Plant Biotechnology Journal* **15,** 257–268 (2017).
81. Lawrenson, T. *et al.* Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. *Genome Biol.* **16,** 258–13 (2015).
82. Garcia-Gimenez, G. *et al.* Targeted mutation of barley (1,3;1,4)-β-glucan synthases reveals complex relationships between the storage and cell wall polysaccharide content. *Plant J.* **104,** 1009–1022 (2020).
83. Feng, Z. *et al.* Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res* **23,** 1229–1232 (2013).
84. Zhang, Y. *et al.* Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat Commun* **7,** (2016).
85. Hamada, H. *et al.* Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat. *Scientific Reports* **8,** (2018).
86. Woo, J. W. *et al.* DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology* **33,** 1162–1164 (2015).
87. Poddar, S., Tanaka, J., Cate, J. H. D., Staskawicz, B. & Cho, M.-J. Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays. *Plant Methods* **16,** 151–11 (2020).
88. Brandt, K. M., Gunn, H., Moretti, N. & Zemetra, R. S. A streamlined protocol for wheat (Triticum aestivum) protoplast isolation and transformation with CRISPR-Cas ribonucleoprotein complexes. *Front. Plant Sci.* **11,** 769 (2020).
89. Sant'Ana, R. R. A., Caprestano, C. A., Nodari, R. O. & Agapito-Tenfen, S. Z. PEG-Delivered CRISPR-Cas9 ribonucleoproteins system for gene-editing screening of maize protoplasts. *Genes (Basel)* **11,** 1029 (2020).
90. Zhang, Y., Iaffaldano, B. & Qi, Y. CRISPR ribonucleoprotein-mediated genetic engineering in plants. *Plant Commun* **2,** 100168 (2021).
91. in *Atlas of Oral Microbiology* (eds. Zhou, X. & Li, Y.) 95–107 (Academic Press, 2015). doi:10.1016/B978-0-12-802234-4.00005-7
92. Malzahn, A. A. *et al.* Application of CRISPR-Cas12a temperature sensitivity for improved genome editing in rice, maize, and Arabidopsis. *BMC Biol.* **17,** 9–14 (2019).
93. Yu, J., Tu, L., Subburaj, S., Bae, S. & Lee, G.-J. Simultaneous targeting of duplicated genes in Petunia protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins. *Plant Cell Rep.* **40,** 1037–1045 (2021).

94. Faris, J. D. *et al.* A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 13544–13549 (2010).
95. Kariyawasam, G. K. *et al.* The Parastagonospora nodorum necrotrophic effector SnTox5 targets the wheat gene Snn5 and facilitates entry into the leaf mesophyll. *New Phytologist* (2021). doi:10.1111/nph.17602
96. Friesen, T. L. & Faris, J. D. Characterization of Effector-Target Interactions in Necrotrophic Pathosystems Reveals Trends and Variation in Host Manipulation. *Annu Rev Phytopathol* **59,** 77–98 (2021).
97. Rodríguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E. & Lippman, Z. B. Engineering quantitative trait variation for crop improvement by genome editing. *Cell* **171,** 470–480.e8 (2017).
98. Liu, Z. *et al.* SnTox3 acts in effector triggered susceptibility to induce disease on wheat carrying the Snn3 gene. *PLoS Pathog* **5,** e1000581 (2009).
99. Friesen, T. L. & Faris, J. D. Characterization of plant-fungal interactions involving necrotrophic effector-producing plant pathogens. *Methods Mol Biol* **835,** 191–207 (2012).
100. Poddar, S. *et al.* Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays. *Plant Methods* **16**, 151 (2020).