

1 **Impact of temperature and time on DNA-free Cas9-ribonucleoprotein mediated gene  
2 editing in wheat protoplasts and immature embryos**

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46

47 **Summary**

48

49 The advancement of precision engineering for crop trait improvement is important in the face of  
50 rapid population growth, climate change, and disease. To this end, targeted double-stranded  
51 break technology using RNA-guided Cas9 has been adopted widely for genome editing in plants.  
52 *Agrobacterium* or particle bombardment-based delivery of plasmids encoding Cas9 and guide  
53 RNA (gRNA) is common, but requires optimization of expression and often results in random  
54 integration of plasmid DNA into the plant genome. Recent advances have described gene editing  
55 by the delivery of Cas9 and gRNA as pre-assembled ribonucleoproteins (RNPs) into various  
56 plant tissues, but with moderate efficiency in resulting regenerated plants. In this report we  
57 describe significant improvements to Cas9-RNP mediated gene editing in wheat. We  
58 demonstrate that Cas9-RNP assays in protoplasts are a fast and effective tool for rational  
59 selection of optimal gRNAs for gene editing in regenerable immature embryos (IEs), and that  
60 high temperature treatment enhances gene editing rates in both tissue types. We also show that  
61 Cas9-mediated editing persists for at least 14 days in gold particle bombarded wheat IEs. The  
62 regenerated edited wheat plants in this work are recovered at high rates in the absence of  
63 exogenous DNA and selection. With this method, we produce knockouts of a set of three  
64 homoeologous genes and two pathogenic effector susceptibility genes that result in insensitivity  
65 to corresponding necrotrophic effectors produced by *Parastagonospora nodorum*. The  
66 establishment of highly efficient, DNA-free gene editing technology holds promise for  
67 accelerated trait diversity production in an expansive array of crops.

68

69 **Keywords**

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71 wheat, gene editing, Cas9, ribonucleoproteins, DNA-free, temperature treatment, protoplasts,  
72 immature embryos, necrotrophic effector sensitivity

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## 90 Introduction

91  
92 Amidst a rapidly growing population and threats posed by climate change and disease, there  
93 exists a need for the advancement of crop biotechnology to increase the speed and precision of  
94 crop varietal development. Cas9 has emerged as a plant gene editing tool of choice for its  
95 accuracy and programmability to engineer allelic diversity for beneficial traits to support global  
96 food security. Guided by RNA, Cas9 efficiently makes sequence-specific double-stranded breaks  
97 in genomic DNA (Jinek et al. 2012). The host's double-stranded break repair mechanisms are  
98 then elicited. Non-homologous end joining (NHEJ), the predominant and often error prone  
99 pathway in plants, can lead to insertions or deletions (indels) at the Cas9 cut site upon repair  
100 (Puchta 2005). Exploitation of this system allows for targeted knockout of endogenous genes.

101  
102 Cas9 and guide RNA (gRNA) encoding plasmid DNA systems have been developed and  
103 delivered to plant and major crop species including *Arabidopsis* (Li et al. 2013), potato (Wang et  
104 al. 2015; Butler et al. 2015), tomato (Brooks et al. 2014; Lor et al. 2014), soybean (Jacobs et al.  
105 2015), maize (Svitashov et al. 2015; Char et al. 2017), barley (Lawrenson et al. 2015; Garcia-  
106 Gimenez et al. 2020), rice (Feng et al. 2013), and wheat (Wang et al. 2014) by *Agrobacterium*  
107 *tumefaciens* or particle bombardment. These methods rely on random integration of Cas9-gRNA  
108 cassettes into the genome, and optimization of expression for each plant system. As a result, the  
109 gene editing process is encumbered by variables such as promoter and terminator choice when  
110 cloning constructs and copy number and integration location of transgenes upon transformation.  
111 Additionally, gene editing by these methods raise transgenic regulatory concerns. Regulation  
112 aside, transgenes can often be segregated away through breeding, but the process is laborious,  
113 time consuming, and particularly difficult for plants with complex genomes. Moreover, crops  
114 with lengthy generation times or those that are vegetatively propagated, such as cassava and  
115 banana, cannot be bred to segregate transgenes. There have been reports in which plant gene  
116 editing has been achieved by transient expression of Cas9 and gRNA (Zhang et al. 2016;  
117 Hamada et al. 2018), however full experimental control over the fate of transgene integration and  
118 tracking has not been achieved. For these reasons, there is a clear need for advances in DNA-free  
119 genome editing technology.

120  
121 The direct delivery of preassembled Cas9-gRNA ribonucleoproteins (RNPs) is one such  
122 technology and has been demonstrated in various plant protoplast systems to induce targeted  
123 mutations (Woo et al. 2015; Malnoy et al. 2016; Poddar et al. 2020; Brandt et al. 2020; Sant'Ana  
124 et al. 2020). Some have produced edited plants arising from the transfected single cells. However  
125 regeneration of wheat and other crop plant protoplasts is not feasible with current methods.  
126 Cas9-RNP based editing of maize (Svitashov et al. 2016), rice (Banakar et al. 2019), and wheat  
127 (Liang et al. 2017) regenerable embryos by biolistics has also been reported. Gold particles  
128 coated with Cas9-RNPs are bombarded with high pressure into immature embryos (IEs) that are  
129 ultimately regenerated into plants through tissue culture. Co-delivery of DNA vectors with  
130 selective markers or helper genes along with Cas9-RNPs have been utilized to improve editing  
131 efficiency (Svitashov et al. 2016; Banakar et al. 2019). In the absence of selection, however,  
132 editing rates have generally been low.

133  
134 The use of Cas9-RNPs to generate edited plants provides unique benefits. Because the gene  
135 editing reagents are delivered as pre-assembled complexes, researchers do not need to optimize

136 DNA vectors, the host plant tissue does not bear the burden of transcribing or translating Cas9 or  
137 gRNA, and breeding for segregation is unnecessary due to the absence of transgenes.

138 Additionally, the Cas9-RNPs, which exist in a finite amount in the target tissue, are ultimately  
139 degraded by endogenous proteases and nucleases. However, there remains room to improve the  
140 editing pipeline and increase efficiency.

141  
142 Low rates of Cas9 mediated editing in plant tissue may indicate that the endonuclease is not  
143 reaching its full potential due to suboptimal environmental conditions. For example, studies  
144 across organisms including *Arabidopsis*, citrus (LeBlanc et al. 2018), and wheat (Milner et al.  
145 2020) have shown that Cas9 generates more targeted indels at elevated temperatures.

146  
147 Here, we present advances in Cas9-RNP based gene editing in the global food crop, wheat  
148 (*Triticum aestivum*). To determine if temperature can be harnessed to enhance Cas9-RNP  
149 mediated editing, we explore the effects of heat treatment on transfected wheat protoplasts and  
150 IEs. We examine the relationship of editing efficiency between non-regenerable protoplasts and  
151 regenerable IEs and monitor the rate of editing over time. We demonstrate that treatment at  
152 elevated temperatures increases gene editing efficiency in both tissue systems and find that the  
153 RNP transfection technique of gold particle bombardment results in sustained editing of tissue at  
154 least 14 days after bombardment. We also find that editing rates in protoplasts correlate linearly  
155 with editing rates in IEs. Therefore, rapid *in vivo* protoplast assays can be instituted as a standard  
156 gene editing pipeline step to select the most effective gRNAs for IE gene editing and  
157 regeneration. Lastly, we regenerate wheat plants edited via Cas9-RNP biolistic transfection. As a  
158 proof of method, we simultaneously target three wheat homoeologous orthologs of a rice gene,  
159 *Pi21*(Os04g0401000), and successfully generate lines with knockouts in all copies. We also  
160 target wheat genes *Tsn1* and *Snn5*, producing lines that are insensitive to the *Parastagonospora*  
161 *nodorum* pathogenic effectors SnToxA and SnTox5 and establish DNA and selection-free Cas9-  
162 RNP mediated editing as an efficient and feasible technique for generating targeted gene  
163 knockouts in wheat.

164

## 165 **Results**

166

### 167 **Cas9-RNP transfection and the effect of temperature in wheat protoplast gene editing**

168

169 We first quantified cell viability after heat treatment of non-transfected protoplasts to determine  
170 the feasibility of testing higher temperatures for wheat protoplast gene editing. Protoplasts were  
171 isolated from partially etiolated wheat seedlings and incubated at 25°C, 30°C, or 37°C for 16  
172 hours followed by 25°C for 8 hours. During the 24-hour period, the protoplasts were monitored  
173 for viability every 8 hours using Evans blue staining and microscopy. Viability of protoplasts  
174 treated at 37°C decreased markedly compared to those treated at 25°C and 30°C (Figure S1) and  
175 suffered from media evaporation. It was therefore concluded that the protoplast gene editing  
176 pipeline was not amenable to a 37°C heat treatment.

177

178 Five single guide RNAs (sgRNAs), *Pi21gD*, *Tsn1g2*, *Tsn1g3*, *Snn5g1*, and *Snn5g2* were selected  
179 and commercially synthesized for this study. To assess the efficacy of the sgRNAs *in vivo*, and to  
180 determine the effect of temperature on wheat protoplast gene editing, Cas9-RNPs were  
181 assembled and transfected into wheat mesophyll protoplasts. Purified Cas9 with a C-terminal

182 double nuclear-localization tag was complexed with sgRNA. The resulting sgRNA-Cas9 RNPs  
183 were transfected into wheat protoplasts using polyethylene glycol (PEG). Transfected protoplasts  
184 were treated at 25°C or 30°C and harvested for genotypic analysis after 24 hours. Editing rates at  
185 the target loci were determined by amplicon next-generation sequencing (NGS). With incubation  
186 at 25°C and 30°C, average editing rates ranged from 2.5-50% and 5.8-62% respectively. Despite  
187 this variability between different sgRNA-Cas9 RNPs, editing efficiency was consistently higher  
188 in protoplasts treated at 30°C compared to 25°C for any given sgRNA (Figure 1), suggesting that  
189 a higher temperature treatment is advantageous to RNP-mediated gene editing in wheat  
190 protoplasts.

191

## 192 **Biostatic Cas9-RNP delivery and the effect of temperature in wheat immature embryo gene 193 editing**

194

195 To determine if a high temperature treatment similarly improves Cas9-RNP based editing in  
196 wheat IEs as it does in protoplasts, RNPs were transfected into IEs by particle bombardment.  
197 The experimental pipeline is summarized in Figure 2a. Single guide RNA and Cas9 were  
198 complexed *in vitro*, adsorbed onto 0.6 µm gold particles, and biolistically delivered with a  
199 helium-pressured particle gun. For each sgRNA and temperature being tested, 30 IEs were  
200 bombarded and incubated at 26°C, 30°C, or 37°C for 16 hours. They were then maintained at  
201 26°C on callus-induction media before inducing regeneration at around 63 days post-  
202 bombardment (dpb). Plasmid DNA was not co-delivered with any of the Cas9-RNPs, and callus  
203 induction and regeneration were performed under selection-free conditions. From each set of 30  
204 RNP-transfected embryos, ten were randomly harvested and pooled for genomic analysis at 14  
205 dpb and again at 48 dpb. The remaining ten embryos were kept for regeneration into M<sub>0</sub> plants.  
206 All independent shoots were isolated and treated as individual M<sub>0</sub> plants. Plants were  
207 transplanted from tissue culture media to soil approximately 100 dpb. Each resulting M<sub>0</sub> plant  
208 was independently genotyped, and the percent tissue edited rate was calculated as the percentage  
209 of mutant alleles among total alleles in the M<sub>0</sub> plant pool. The percentage of plants edited was  
210 also calculated as a percentage of the number of plants with any edit among the number of total  
211 M<sub>0</sub> plants regenerated. All genomic analysis was done by amplicon NGS.

212

213 Elevated temperature treatment of both 30°C and 37°C led to higher percentages of edited tissue  
214 compared to 26°C for all five sgRNA-Cas9 RNPs across all timepoints (Figure 2b). Tissue  
215 editing rates were higher at 48 dpb than at 14 dpb and editing rates in the M<sub>0</sub> regenerant tissue  
216 pool were comparable to those at 48 dpb. From the ten embryos per treatment allowed to  
217 regenerate, 10-40 M<sub>0</sub> plants were produced. Plants with wild type, heterozygous, biallelic, and  
218 homozygous mutations at the target loci were obtained. Editing efficiency in the M<sub>0</sub> regenerants  
219 is summarized in Table 1 and genotypes of each individual edited M<sub>0</sub> regenerant are described in  
220 Table S3.

221

## 222 **Cas9-RNP mediated editing is sustained over time**

223

224 Notably, gene editing rates were more than doubled, regardless of temperature treatment, in  
225 tissue assayed at 48 dpb compared to 14 dpb (Figure 2b). To further investigate the difference in  
226 editing rates over time, the number of unique mutant alleles was determined at the 14 and 48 dpb  
227 timepoints. With minimal exception, there were more unique mutant alleles at 48 dpb compared

228 to 14 dpb (Figure 3a, Figure S2).

229  
230 An additional 50 IEs were bombarded with Snn5g1-Cas9 RNP to determine the length of time  
231 that Cas9 remains present in biolistically transfected tissue. Western blot analysis was performed  
232 with 10-embryo tissue samples taken 0, 2, 7, and 14 dpb. Given the finite amount of Cas9 protein  
233 delivered by RNP bombardment and rapid cell division and growth in each IE over time, we  
234 normalized the experiment by volume extracted from total tissue originating from ten IEs at any  
235 given timepoint, rather than total protein extracted. Cas9 was detected in tissue from all four  
236 timepoints with decreasing band intensity over time (Figure 3b). Cas9 was not detected in  
237 embryos that were not subjected to bombardment of Cas9-RNPs. Due to the large mass of tissue  
238 from exponential growth of callus from IEs, it was not feasible to extract protein from and  
239 perform Western blot analysis on ten-embryo 48 dpb samples. Taken together, these results  
240 suggest that Cas9 mediated editing activity is sustained over the course of at least 14 days after  
241 biotic delivery of Cas9-RNPs into immature wheat embryos. When using this method, the  
242 degradation of Cas9 protein in the target tissue is not as rapid as previously hypothesized (Kim et  
243 al. 2014), and evaluation of editing efficiency should occur 14 to 48 dpb for increased accuracy.

244  
245 **Relative editing rates in protoplasts correlate linearly with editing rates in M<sub>0</sub> regenerants**  
246 **from bombarded immature embryos**

247  
248 The different sgRNA-Cas9 RNPs used in this study conferred different levels of efficacy in both  
249 PEG transfected protoplasts and biolistically transfected embryos. To determine whether the  
250 editing rates in the two tissue systems correlated with one another, each sgRNA-Cas9 RNP's  
251 average editing efficiency in 30°C treated protoplasts was plotted against its editing efficiency in  
252 48 dpb 30°C treated bombarded IEs as well as the M<sub>0</sub> 30°C treated regenerant tissue pool. A  
253 linear regression model was applied to the data, revealing a positive linear correlation with  
254  $R^2=0.744$  and  $R^2=0.994$ , respectively (Figure 4). Though a survey of a greater number of  
255 sgRNAs would strengthen this association, the present data suggest that editing efficiency in  
256 protoplasts can be predictive of editing efficiency in IEs. Given the positive correlation between  
257 RNP-mediated editing rates in protoplasts and in biolistically transfected IEs, it can be beneficial  
258 to first rapidly score the efficiency of various gRNA candidates in protoplasts to optimize for the  
259 highest rate of edited regenerant tissue.

260  
261 **Cas9-RNP mediated knockout of *Parastagonospora nodorum* necrotrophic effector**  
262 **sensitivity genes**

263  
264 The wheat genes *Tsn1* and *Snn5* recognize necrotrophic effectors produced by *Parastagonospora*  
265 *nodorum*, and each exist as single copy genes on the B genome of allohexaploid wheat. In this  
266 study, 20 M<sub>0</sub> *Tsn1* edited plants were produced from 30 transfected embryos maintained for  
267 regeneration. Of those, 14 had heterozygous mutations and 6 had biallelic or homozygous  
268 mutations. Fully expanded secondary leaves of a subset of M<sub>0</sub> *Tsn1* edited plants, M<sub>0</sub> *Tsn1* WT  
269 plants, and Fielder grown from seed were infiltrated with SnToxA expressed in *Pichia pastoris*.  
270 After 72 hours, M<sub>0</sub> heterozygotes, M<sub>0</sub> WT, and Fielder plants had necrotic lesions extending from  
271 the site of infiltration. Meanwhile, M<sub>0</sub> plants with biallelic or homozygous mutations exhibited  
272 no necrosis (Figure 5).

274 Similarly, a total of 24 M<sub>0</sub> *Snn5* edited plants were produced from 30 transfected embryos  
275 maintained for regeneration. Of those, 14 had heterozygous mutations and ten had biallelic or  
276 homozygous mutations. Fully expanded secondary leaves of a subset of M<sub>0</sub> *Snn5* edited plants,  
277 M<sub>0</sub> *Snn5* WT plants, and Fielder grown from seed were infiltrated with SnTox5 containing  
278 culture filtrates. After 72 hours, M<sub>0</sub> heterozygotes with in-frame deletions, M<sub>0</sub> WT, and Fielder  
279 plants exhibited necrotic lesions. Results for M<sub>0</sub> heterozygotes, however, displayed a mixture of  
280 phenotypes ranging from sensitive to insensitive. Two heterozygous plants with an in-frame  
281 deletion on one allele appeared insensitive to SnTox5. Notably, all plants with biallelic or  
282 homozygous mutations leading to premature termination were insensitive to SnTox5 (Figure 6).  
283

284 These results demonstrate that loss-of-function mutations can be introduced to both copies of a  
285 gene within the M<sub>0</sub> generation, leading to insensitivity to agronomically relevant necrotrophic  
286 fungal effectors. M<sub>0</sub> heterozygotes and biallelic plants can be self-fertilized to establish lines with  
287 homozygous deleterious mutations in the susceptibility genes. The biolistic method with 30°C or  
288 37°C heat treatment is highly efficient, and edited plants can be identified from a small number  
289 of regenerants without the use of selection in tissue culture.  
290

## 291 Discussion

292

293 CRISPR-based RNPs have been used for editing in various plant species and tissue types (Zhang  
294 et al. 2021). In this work, we improve upon DNA-free Cas9-RNP technology for genome editing  
295 in wheat. We establish heat treatment as a parameter to increase the rate of editing *in vivo*, show  
296 that particle bombardment-based editing is sustained over more than 14 days, and demonstrate  
297 that results from protoplast assays can be utilized as a proxy for predicting editing rates in  
298 regenerable tissue and as a tool to rank gRNA efficacy. By delivering gene editing reagents as  
299 protein-RNA complexes, several complications associated with *Agrobacterium tumefaciens* and  
300 biolistic DNA vector delivery are avoided.  
301

302 Cas9 from *Streptococcus pyogenes*, a bacterium that grows optimally at 37°C (Zhou & Li 2015),  
303 has been shown to exhibit increased cleavage activity at 37° compared to 22°C *in vitro* (LeBlanc  
304 et al. 2018). Plant protoplast and IE transfections and regeneration are typically performed at  
305 ambient temperatures (25°C and 26°C respectively). Although modulation of temperature has  
306 not been previously performed in protoplast gene editing experiments, an increase in temperature  
307 for DNA-based plant gene editing studies have resulted in higher targeted mutation frequencies  
308 (LeBlanc et al. 2018; Malzahn et al. 2019). The application of temperature treatment to increase  
309 Cas9-RNP mediated editing efficiency in any plant tissue system has not previously been  
310 demonstrated. Here, we found that 16 hours of exposure of Cas9-RNP transfected protoplasts to  
311 30°C markedly increased indel formation at the Cas9 cut site (Figure 1). Similarly, 16 hours of  
312 exposure of Cas9-RNP bombarded IEs to 30°C or 37°C resulted in increased targeted indel  
313 formation. In IEs assayed at 48 dpb we achieved editing rates of 10.4-34.9% with 30°C  
314 treatment, 6.63-24.39% with 37°C treatment, and just 3.36-14.25% with standard 26°C  
315 incubation (Figure 2b). Interestingly, the benefit of increased temperature treatment was  
316 consistent between the two target tissues and across the five different target sites tested. In our  
317 work, there were no discernable defects in regenerability for IEs treated at a higher temperature  
318 compared to the standard 26°C. We detected no positive or negative correlation between  
319 temperature treatment and the number of M<sub>0</sub> plants recovered.

320 Two reports have described the biolistic delivery of Cas9-RNPs into wheat and maize embryo  
321 cells in the absence of DNA and selection (Liang et al. 2017; Svitashov et al. 2016). Both  
322 achieved moderate targeted mutagenesis frequencies in the regenerated plants. We noted that the  
323 studies each assayed for editing efficiency in the IEs 2 dpb and universally achieved <1%  
324 targeted editing. In contrast, the editing efficiencies in regenerated plant tissue were substantially  
325 higher, ranging from 1.3-4.7% (Liang et al. 2017) and 2.4-9.7% (Svitashov et al. 2016). To  
326 investigate this discrepancy between timepoints, we monitored editing efficiency at 14 dpb, 48  
327 dpb, and in the M<sub>0</sub> regenerants in our study. Irrespective of temperature treatment or gRNA  
328 sequence, editing frequencies at 48 dpb were considerably higher than at 14 dpb (Figure 2b).  
329 Percentage of tissue edited in the M<sub>0</sub> plant pool was comparable to that at 48 dpb. The observed  
330 difference in editing efficiency between earlier timepoints and regenerated M<sub>0</sub> plants was  
331 consistent with previous reports (Liang et al. 2017; Svitashov et al. 2016).  
332

333 In mammalian cells, Cas9 was shown to be undetectable 48-72 hours after Cas9-RNP  
334 transfection by nucleofection (Kim et al. 2014). For this reason, it has been thought that  
335 enzymatic degradation of Cas9-RNPs *in vivo* is rapid and that editing must occur within the first  
336 few days of transfection. In the present study, if Cas9-RNPs were fully degraded from the tissue  
337 prior to the 14 dpb timepoint, all gene editing would have had to occur before 14 dpb.  
338 Consequently, approximately the same number of unique alleles would have been expected to be  
339 detected at both 14 dpb and 48 dpb if proliferation of edited and unedited cells occurs at the same  
340 rate. On the contrary, consistently higher rates of mutagenesis as well as a greater number of  
341 unique alleles at the later timepoints were observed at 48 dpb (Figure 2b, Figure 3a, Figure S2),  
342 suggesting that Cas9 may somehow be stabilized for at least 14 days and gradually released  
343 within the wheat IEs after biolistic delivery for sustained editing over time. As further evidence  
344 in support of this hypothesis, Cas9 protein was detected in 10-embryo tissue samples taken 2, 7,  
345 and 14 dpb (Figure 3b). Taken together, these results indicate that Cas9 is maintained in tissue at  
346 least 14 dpb and facilitates sustained and gradual editing of tissue over time when delivered as  
347 Cas9-RNP via gold particle bombardment. Further biochemical exploration is necessary to  
348 understand the mechanism of this Cas9 stabilization and persistent editing.  
349

350

351 Numerous plant protoplast systems have been used for targeted mutagenesis using Cas9-RNPs  
352 (Woo et al. 2015; Malnoy et al. 2016; Shan et al. 2019; Poddar et al. 2020; Brandt et al. 2020;  
353 Sant'Ana et al. 2020; Yu et al. 2021). Although the method is useful for producing Cas9-RNP  
354 edited plants for protoplasts that are amenable to regeneration, most crop plants cannot easily be  
355 regenerated in this manner. Though wheat protoplasts are recalcitrant to regeneration through  
356 existing methodology, protoplasts in the current study prove to be a beneficial screening system.  
357 Cas9-RNP mediated editing rates in protoplasts correlated linearly with editing rates in IEs.  
358 Because biolistic Cas9-RNP transfection of IEs requires significant time, energy, resources, and  
359 commitment, a means for rational selection of gRNA sequences for optimal editing efficiency is  
360 preferred. It is noteworthy that there were major differences in mutation rates for the 5 gRNAs  
361 used in this study. Unfortunately, existing predictive software to select gRNAs often do not  
362 translate upon experimentation. Therefore, when attempting to select the best gRNA to produce  
363 the highest rate of stable editing in regenerable IEs, transient protoplast assays can serve as a  
364 rapid pipeline to rank gRNAs and forecast editing rates in Cas9-RNP bombarded regenerable  
365 tissue.

366

367 The calculation of editing efficiency in M<sub>0</sub> regenerants has the potential to be confusing. To be  
368 explicit in our analysis, we present editing rates of regenerants in two ways. The percentage of  
369 total edited alleles in the M<sub>0</sub> regenerant pool is indicated as “% Tissue edited”, while “% Plants  
370 edited” is the percentage of total edited plants among all the M<sub>0</sub> plants (Figure 2b, Figure 4,  
371 Table 1). The former is meant to compare overall editing efficiency more fairly across tissue  
372 types and timepoints, taking biallelism, homozygosity, and heterozygosity of regenerated plants  
373 into consideration. The latter value is more relevant for evaluating the method’s ability to  
374 produce individual plants with gene edits.

375

376 The gene *Pi21* was first characterized in rice (*Oryza sativa*) as a negative regulator of resistance  
377 for blast disease (Fukuoka et al. 2009). We identified putative orthologs in wheat that consisted  
378 of three homoeologous genes. The functionality of wheat *Pi21* has not been formally assessed  
379 but may potentially play a role in disease susceptibility. Wheat *Pi21* was selected as a target to  
380 demonstrate the DNA-free Cas9-RNP gene editing method in a gene present in all three diploid  
381 subgenomes (AABBDD). *Pi21gD* was designed to simultaneously target all six alleles. Despite  
382 the genetic complexity, we were able to regenerate plants with biallelic or homozygous  
383 mutations across all three subgenomes for a full variety of genotypes including two with biallelic  
384 or homozygous triple mutant edits within the M<sub>0</sub> generation (Table S3).

385

386 The wheat genes *Tsn1* and *Snn5* recognize the *Parastagonospora nodorum* pathogenic effectors  
387 SnToxA and SnTox5, respectively (Faris et al. 2010; Kariyawasam et al. 2021). *Tsn1* is a gene  
388 with resistance gene-like features including protein kinase, nucleotide binding, and leucine-rich  
389 repeats, and the ToxA necrotrophic effector is produced by at least three economically important  
390 fungal pathogens of wheat (Friesen & Faris 2021). *Snn5* belongs to a different class and contains  
391 protein kinase and major sperm protein domains (details regarding the cloning and  
392 characterization of *Snn5* will be published in the future; K.L.D. Running and J.D. Faris, personal  
393 communication), but like *Tsn1*, it functions as a target for a necrotrophic effector leading to  
394 disease susceptibility (Kariyawasam et al. 2021). Therefore, *Tsn1* and *Snn5* are practical targets  
395 for disruption via DNA-free gene editing. Using DNA-free biolistic delivery of Cas9-RNPs, we  
396 successfully generated plants with heterozygous, biallelic, and homozygous mutations within the  
397 M<sub>0</sub> generation from a mere ten IEs per treatment. Biallelic and homozygous mutants of *Tsn1* and  
398 *Snn5* were demonstrated to be insensitive to SnToxA and SnTox5, respectively. Due to the high  
399 rate of editing, particularly using *Snn5g1* and *Snn5g2* with 30°C and 37°C heat treatment,  
400 screening of M<sub>0</sub> plants for edits was fully feasible. Contrary to previous reports, a selection  
401 scheme can reasonably be foregone with Cas9-RNP mediated editing so long as gRNAs are pre-  
402 tested in protoplasts and deemed to be highly effective.

403

404 In summary, heat treatment enhancement of Cas9-RNP mediated wheat editing combined with a  
405 protoplast-based approach to select optimal gRNAs, and findings that editing is sustained for  
406 more than 2 weeks advances this DNA and selection-free gene editing approach in crops. Given  
407 the persistence of Cas9 in bombarded tissue, additional work with increased length or punctuated  
408 exposure to heat, beyond 16 hours, throughout callus induction may further augment the benefit  
409 of heat treatment. The success of this method in targeting single loci warrants exploration of  
410 furthering the technique to multiplexing. In addition to knocking out genes, editing via Cas9-  
411 RNPs can conceivably be applied to generating allelic series by targeting non-coding genomic

412 regions such as promoters (Rodríguez-Leal et al. 2017). The presented advancement to this  
413 technology can be applied to numerous crops that are amenable to particle bombardment and  
414 encourages the establishment of tissue culture and regeneration protocols in crop species that are  
415 vegetatively propagated.

416

## 417 **Materials and Methods**

418

### 419 **Plant material**

420

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

423

### 424 **Cas9-gRNA RNP assembly**

425

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

430

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

435

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

439

440 The resultant RNP mixtures were stored on ice until transfection.

441

### 442 **Protoplast isolation and transfection**

443

444 Partially etiolated seedlings were used as donor tissue for protoplast isolation. Seeds were  
445 surface sterilized in 20% (v/v) bleach and rinsed in sterile water. Seedlings were grown under  
446 sterile conditions on wet filter paper in the dark for 12-14 days at 25°C with exposure to ambient  
447 light for 6 hours every 5 days. Wheat protoplasts were isolated from the donor tissue using a  
448 previously described method (Shan et al. 2014). For each transfection 25  $\mu$ l of Cas9-gRNA RNP  
449 mixture, as defined above, were added to  $5 \times 10^5$  protoplasts. PEG-meditated transfection was  
450 performed as described in the literature (Shan et al. 2014). Protoplasts were harvested 24 hours  
451 post-transfection for analysis.

452

### 453 **Gold particle preparation for bombardment**

454

455 Cas9-RNPs were precipitated onto 0.6  $\mu$ m gold particles (#1652262, Bio-Rad, Hercules, CA)  
456 using the cationic lipid polymer TransIT-2020 (Mirus, Madison, WI) as previously described  
457 (Svitashov et al. 2016), with modifications. Briefly, for each 30-IE transfection, 40  $\mu$ l Cas9-RNP

458 mixture, as described above, was mixed gently with 20  $\mu$ l sterile gold particles (10  $\mu$ g  $\mu$ l $^{-1}$  water  
459 suspension) and 1  $\mu$ l TransIT-2020 and incubated on ice for 20 min. The Cas9-RNP coated gold  
460 particles were pelleted in a mini microcentrifuge at 2,000g for 30 s. The supernatant was  
461 removed, and the gold particles were resuspended in 20  $\mu$ l of sterile water by brief sonication.  
462 The coated gold particles were immediately applied to 2 macrocarriers (10  $\mu$ l each) by spotting  
463 numerous small drops and allowed to air dry in a laminar flow hood. For a single transfection,  
464 each 30-IE set was bombarded twice using the 2 prepared macrocarriers.  
465

#### 466 **Immature embryo bombardment and regeneration**

467

468 Plants were grown at 24°C, 16-hour days and 15°C, 8-hour nights under light intensity of 130  
469  $\mu$ mol m $^{-2}$ s $^{-1}$ . Immature seeds containing IEs, sized 1.7-2.2 mm were harvested from wheat spikes  
470 10-13 days after flowering, surface sterilized in 20% (v/v) bleach with one drop of Tween 20 and  
471 triple rinsed with sterile water, followed by extraction of the IEs. The IEs were placed on DBC3  
472 media (Cho et al. 1998), scutellum side up and incubated overnight at 26°C prior to biolistic  
473 transfection. Four hours prior to bombardment, IEs were placed on 55 mm filter paper in the  
474 center of DBC3 osmoticum media containing 0.2 M mannitol and 0.2 M sorbitol (Cho et al.  
475 2000). Using two prepared microcarriers holding Cas9-RNP coated gold microparticles, IEs  
476 were shot twice using the PDS-100/He gene gun (Bio-Rad, Hercules, CA) with rupture pressure  
477 of 1100 psi. The bombarded IEs were transferred from the filter paper directly to the media  
478 below and incubated at 26°C, 30°C, or 37°C for 16 hours. IEs were transferred to standard DBC3  
479 media in dim light (10  $\mu$ mol m $^{-2}$  s $^{-1}$ ) at 26°C for 9 weeks with subculturing as needed. Callus  
480 tissue originating from each IE was transferred to DBC6 media for regeneration (Cho et al.  
481 2015). Resultant plantlets were transferred to rooting media and incubated in high light (90  $\mu$ mol  
482 m $^{-2}$  s $^{-1}$ ) at 26°C and grown to 4-6 inches before being transplanted to soil.  
483

#### 484 **Amplicon next generation sequencing analysis**

485

486 To determine mutation rates by amplicon sequencing, PCR was performed with target-specific  
487 primers (Table S1), amplifying approximately 225 bp around the cut site using Phusion High  
488 Fidelity (New England Biolabs, Ipswich, MA) polymerase. Primers contained a 5'-stub  
489 compatible with Illumina NGS library preparation. PCR products were ligated to Illumina  
490 TruSeq adaptors and purified. Libraries were prepared using a NEBNext kit (Illumina, San  
491 Diego, CA) according to the manufacturer's guidelines. Samples were deep sequenced on an  
492 Illumina iSeq at 200 bp paired-end reads to a depth of approximately 10,000 reads per sample.  
493 Cortado (<https://github.com/staciawymann/cortado>) was used to analyze editing outcomes.  
494 Briefly, reads were adapter trimmed then merged using overlap to single reads. These joined  
495 reads were then aligned to the target reference sequence. Editing rates are calculated by counting  
496 any reads with an insertion or deletion overlapping the cut site or occurring within a 3 bp  
497 window on either side of the cut site. SNPs occurring within the window around the cut site are  
498 not counted. Total edited reads are then divided by the total number of aligned reads to derive  
499 percent edited.  
500

501

#### 502 **Western blot**

503 Total plant tissue originating from 10 IEs at different timepoints were frozen in LN2, ground to a  
504 fine powder by mortar and pestle, and resuspended in 200  $\mu$ l 2x Laemmli Sample Buffer (Bio-  
505 Rad, Hercules, CA) with 2-mercaptoethanol. Samples were boiled for 5 min, and the total  
506 soluble protein extracts (25  $\mu$ l or 40  $\mu$ l per well) were separated on 4-20% Mini-PROTEAN  
507 TGX precast polyacrylamide gels (Bio-Rad, Hercules, CA) and subsequently transferred to a  
508 0.45  $\mu$ m nitrocellulose membrane (GVS, Sanford, ME). For detection of Cas9 protein, anti-  
509 CRISPR/Cas9 C-terminal mouse monoclonal antibody (SAB4200751; Sigma-Aldrich, St. Louis,  
510 MO) and ProSignal Dura ECL Reagent (Genesee Scientific, San Diego, CA) were used.  
511 PageRuler Plus Prestained Protein Ladder (10–250 kDa, Thermo Fisher, Waltham, MA) was  
512 used as a molecular weight marker, and Cas9 protein with a C-terminal double nuclear-  
513 localization tag (QB3 Macrolab, University of California, Berkeley) was used as a positive  
514 control.

515

## 516 **Production of SnToxA**

517

518 *SnToxA* was expressed in the *Pichia pastoris* yeast strain X33 (Liu et al. 2009) and cultured in  
519 yeast peptone dextrose broth (10 g yeast extract, 20 g peptone, 100 ml 20% dextrose in 900 ml  
520 distilled water) for 48 hours at 30 °C. Culture filtrate was harvested and filtered through a 0.45  
521  $\mu$ m HVLP filter membrane (Merk Millipore Ltd., Cork, Ireland) and dialyzed overnight against  
522 water using 3.5 kDa molecular weight cut off Snake Skin dialysis tubing (Thermo Scientific, IL,  
523 USA). Dialyzed filtrate was loaded onto a HiPrep SP XL 16/10 cation exchange column (GE  
524 Healthcare Piscataway, NJ). Unbound protein was washed off the column using a 20 mM sodium  
525 acetate (pH 5.0) buffer prior to a gradient elution of SnToxA using a buffer consisting of 300  
526 mM sodium chloride and 20 mM sodium acetate (pH 5.0). Fractions that contained SnToxA  
527 were collected and frozen prior to lyophilizing to increase the concentration of SnToxA.  
528 Lyophilized samples were dissolved in a buffer consisting of 5 mM MOPS sodium salt (Alfa  
529 Aesar, MA, USA) and water, prior to infiltration into the plants.

530

## 531 **Production of SnTox5**

532

533 *P. nodorum* strain Sn79+Tox5-3, generated by transforming *SnTox5* in to the avirulent *P.*  
534 *nodorum* strain Sn79-1087 (Kariyawasam et al. 2021), was used to prepare the culture filtrates  
535 containing SnTox5 as previously described (Friesen & Faris 2012) with minor modifications. In  
536 brief, Sn79+Tox5-3 was grown on V8-potato dextrose agar medium till spores were released  
537 from pycnidia. The plates were flooded with 10 ml of sterile distilled water, and 500  $\mu$ l of spore  
538 suspension was used to inoculate 60 ml of liquid Fries medium (5 g ammonium tartrate, 1 g  
539 ammonium nitrate, 0.5 g magnesium sulfate, 1.3 g potassium phosphate [dibasic], 3.41 g  
540 potassium phosphate [monobasic], 30 g sucrose, 1 g yeast extract in 1000 ml of distilled water).  
541 Cultures were grown on an orbital shaker at 100 rpm for a week prior to two weeks of stationary  
542 growth under dark conditions at room temperature. Culture filtrates were filtered through a layer  
543 of Miracloth (EMD Millipore Corp, MA, USA) and were concentrated 5-fold using Amicon  
544 Ultracel – 3K centrifugal filters (Merk Millipore Ltd., Cork, Ireland). Culture filtrates were  
545 diluted in a 1:1 ratio with sterile water prior to infiltration into the plants.

546

547

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549

550

## 551 Necrotrophic effector infiltrations

552

553 Infiltrations with SnToxA and SnTox5 containing culture filtrates were conducted as previously  
554 described (Friesen & Faris 2012). Three infiltrations were performed per plant, and sensitivity  
555 was evaluated on a binary scale at 3 days post infiltration.

556

557

## 558 Author Contributions

559

560 SP conceived and designed the experiments, analyzed the data, prepared the tables and figures,  
561 and wrote the manuscript with input from all co-authors. SP and JT performed the experiments.  
562 KLDR, GKK, JDF, TLF, and M-JC provided critical reagents, information, and discussion.  
563 JHDC and BS supervised the work. All authors critically reviewed and edited the manuscript.

564

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566

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571 California at Berkeley.

572

573

## 574 Conflict of Interest

575

576 This research was conducted in the absence of any commercial or financial relationships that  
577 could be construed as a potential conflict of interest.

578

## 579 Tables

580

### 581 Table 1

582 Summary of editing outcomes in *Pi21*, *Tsn1*, and *Snn5* targeted M<sub>0</sub> plants. Data for *Pi21* is  
583 broken down by subgenome. *Tsn1* and *Snn5* are only present on subgenome B. "% Tissue  
584 Edited" indicates the percentage of edited alleles among the total alleles analyzed from the M<sub>0</sub>  
585 pools. "% Plants Edited" indicates the percentage of plants with any level of editing among the  
586 total plants analyzed from the M<sub>0</sub> pools.

587

## 588 Figures

589

### 590 Figure 1

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

594

### 595 Figure 2

595 Cas9-RNP particle bombardment and temperature treatment of wheat immature embryos (IEs).  
596 (a) A schematic of the particle bombardment and editing efficiency assay pipeline. (b) Targeted  
597 editing efficiency of gRNA-Cas9 ribonucleoproteins with different temperature treatments in IEs  
598 across time points. Five gRNAs, Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were  
599 bombarded independently into IEs. Tissue pools at 14 dpb and 48 dpb consisted of 10 randomly  
600 chosen initially bombarded IEs. Editing efficiency for M<sub>0</sub> plants is based on aggregate data from  
601 all independently genotyped M<sub>0</sub> plants that emerged from 10 randomly chosen initially  
602 bombarded IEs. Percent tissue edited is defined as the percentage of tissue with insertions or  
603 deletions within 2 bp of the target cleavage site out of the total tissue pool.

604

### 605 **Figure 3**

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

612

### 613 **Figure 4**

614 Correlation plot between targeted editing efficiency of gRNA-Cas9 RNPs in protoplasts and  
615 immature embryos (IEs) at (a) 48 dpb and in (b) M<sub>0</sub> plants treated at 30°C.

616

### 617 **Figure 5**

618 SnToxA assay in *Tsn1* targeted M<sub>0</sub> regenerants. (a) Fielder control grown from seed. (b-m)  
619 independent M<sub>0</sub> regenerants with (b, c) homozygous wildtype; (d-h) heterozygous (d) -2; (e) -5;  
620 (f) -31; (g) -1; (h) +1; and (i-m) biallelic or homozygous mutant (i) -2, -5; (j) -2, -2, (k) -1, -1; (l)  
621 -2, -2; (m) -1, -1 genotypes. Mutation notation is as follows: a positive number, +, indicates the  
622 number of bases inserted, a negative number, -, indicates the number of bases deleted.

623

### 624 **Figure 6**

625 SnTox5 assay in *Snn5* targeted M<sub>0</sub> regenerants. (a) Fielder control grown from seed. (b-m)  
626 independent M<sub>0</sub> regenerants with (b) homozygous wildtype; (c-d) heterozygous in-frame mutant:  
627 (c) -3; (d) -6; (e-h) heterozygous mutant: (e) -5; (f) +20; (g) +2-1; (h) -4; (i-m) biallelic or  
628 homozygous mutant: (i) -11, -4; (j) -8, -2; (k) -10, -10; (l) +1, -2; (m) -5, -1 genotypes. Mutation  
629 notation is as follows: a positive number, +, indicates the number of bases inserted, a negative  
630 number, -, indicates the number of bases deleted.

631

## 632 **Supporting Information**

633

### 634 **Table S1**

635 gRNA target sequences.

636

### 637 **Table S2**

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

640

641 **Table S3**

642 Genotypes of all edited M<sub>0</sub> plants obtained. + indicates the number of base pairs inserted, -  
643 indicates the number of base pairs deleted.

644

645 **Figure S1**

646 Protoplast viability curve. N=3. Error bars indicate SEM.

647

648

649

650 **Figure S2**

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

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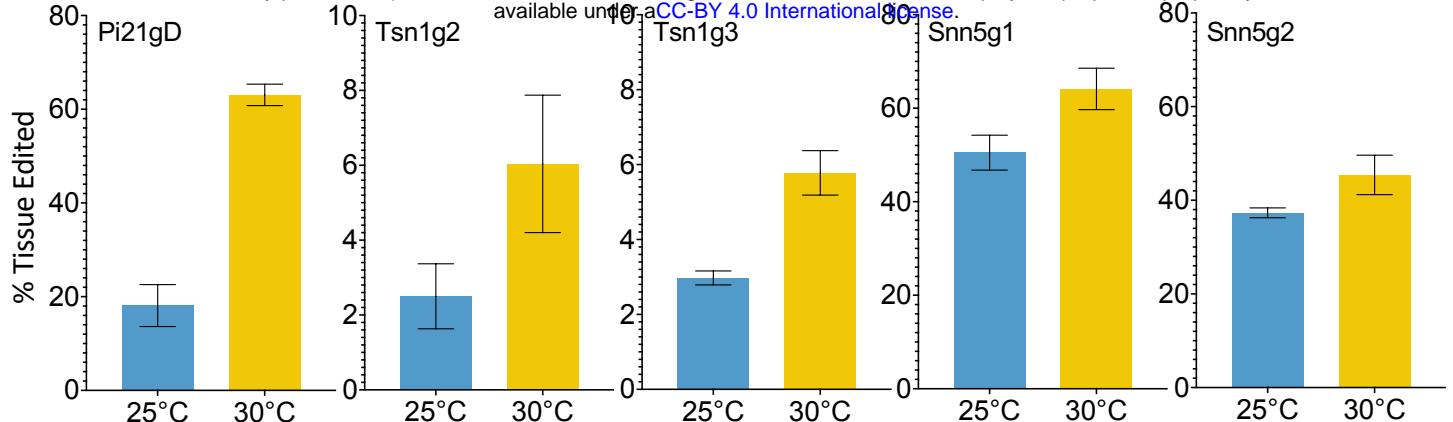
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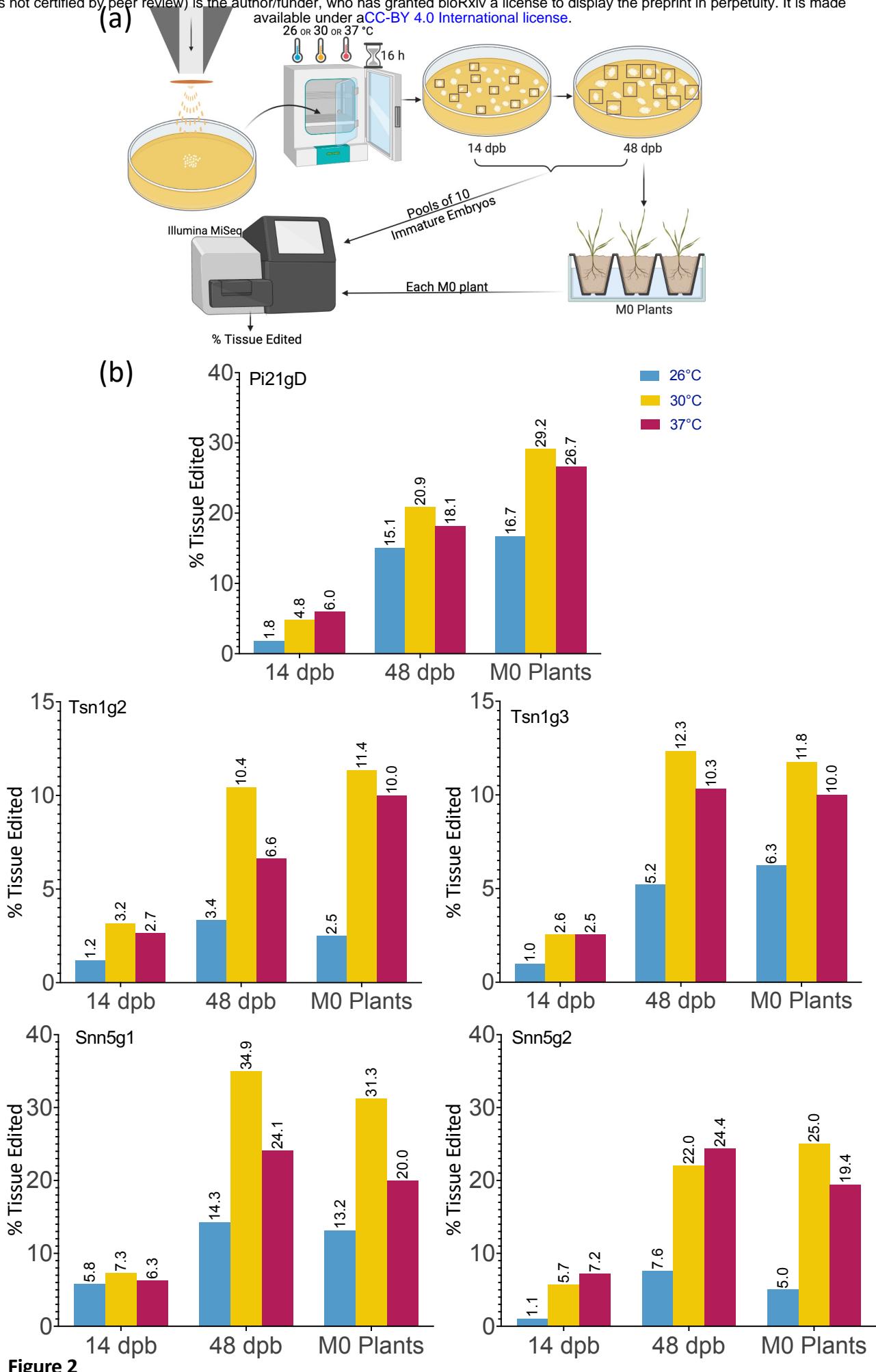
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Temperature Treatment	Immature embryos regenerated	Genome A			Genome B			Genome D			% Tissue Edited	% Plants Edited
		Total MO Plants	Edited Plants	Heterozygous	Biallelic or Homozygous	Heterozygous	Biallelic or Homozygous	Heterozygous	Biallelic or Homozygous			
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 MO 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	

**Table 1**

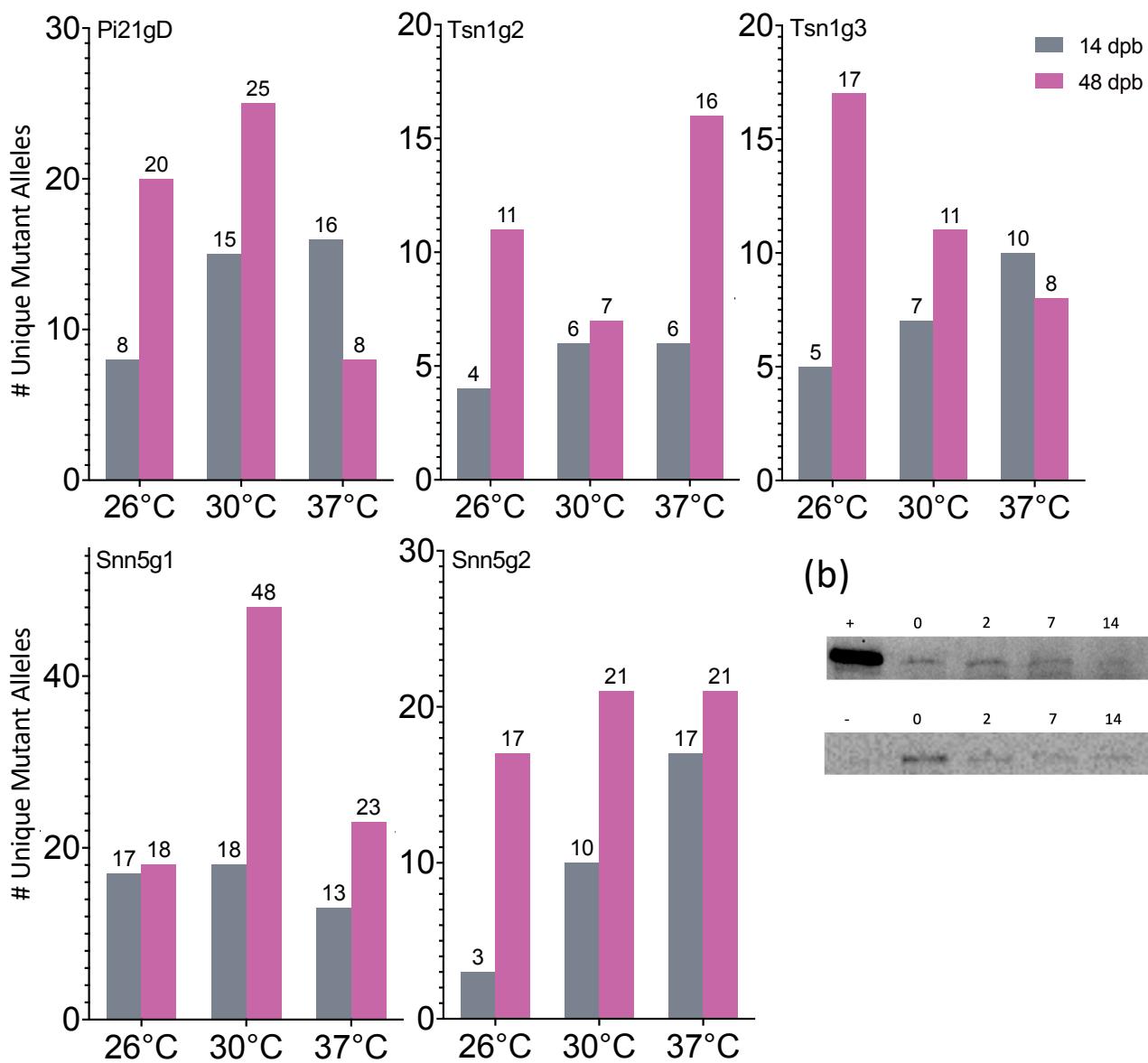


**Figure 1**



**Figure 2**

(a)



(b)

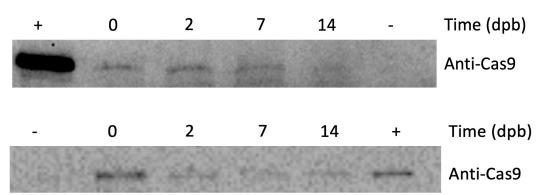
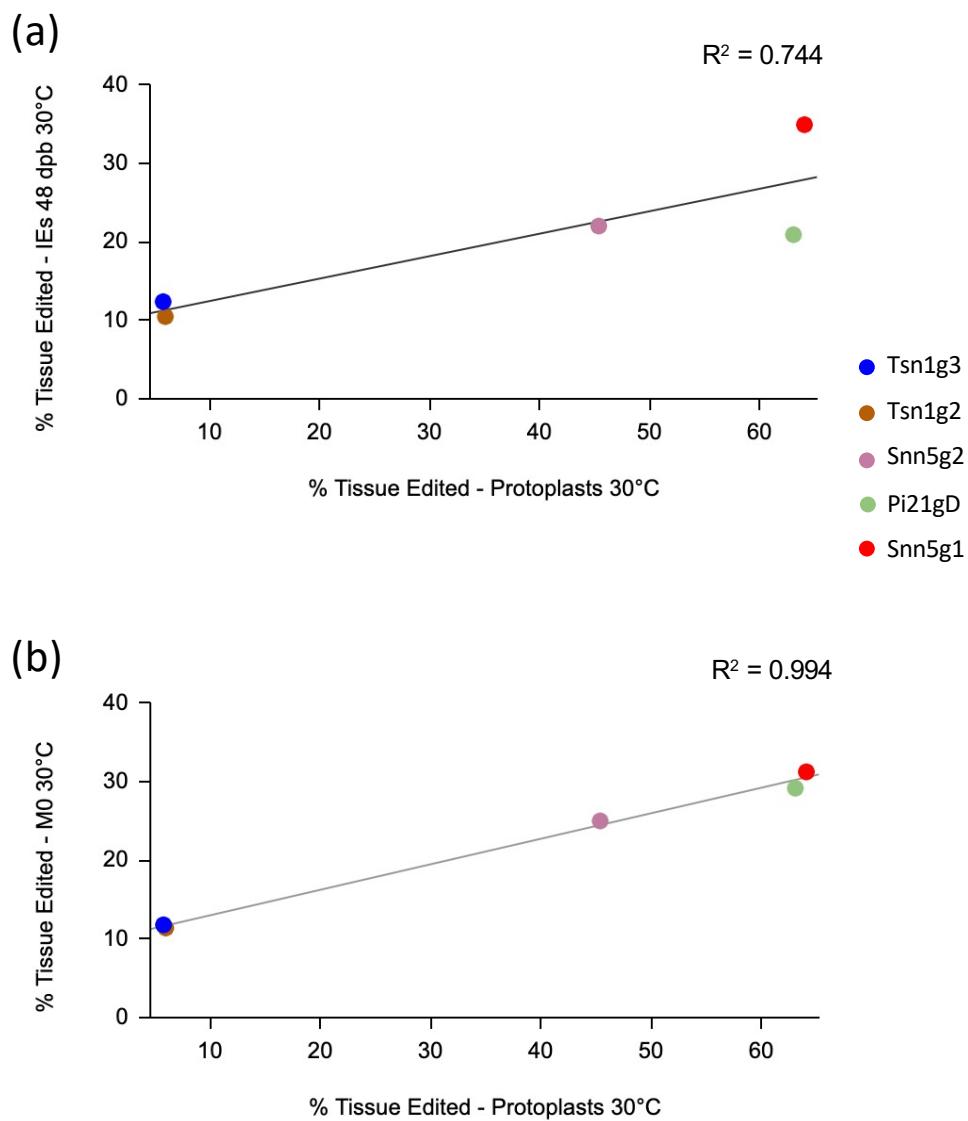
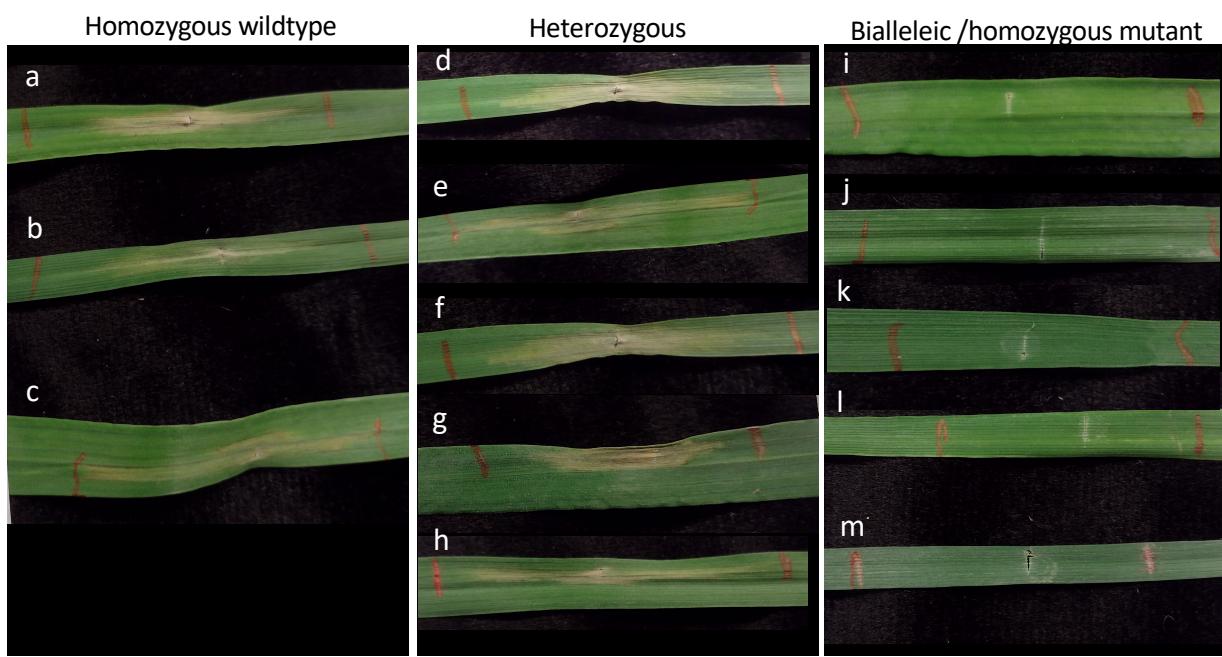


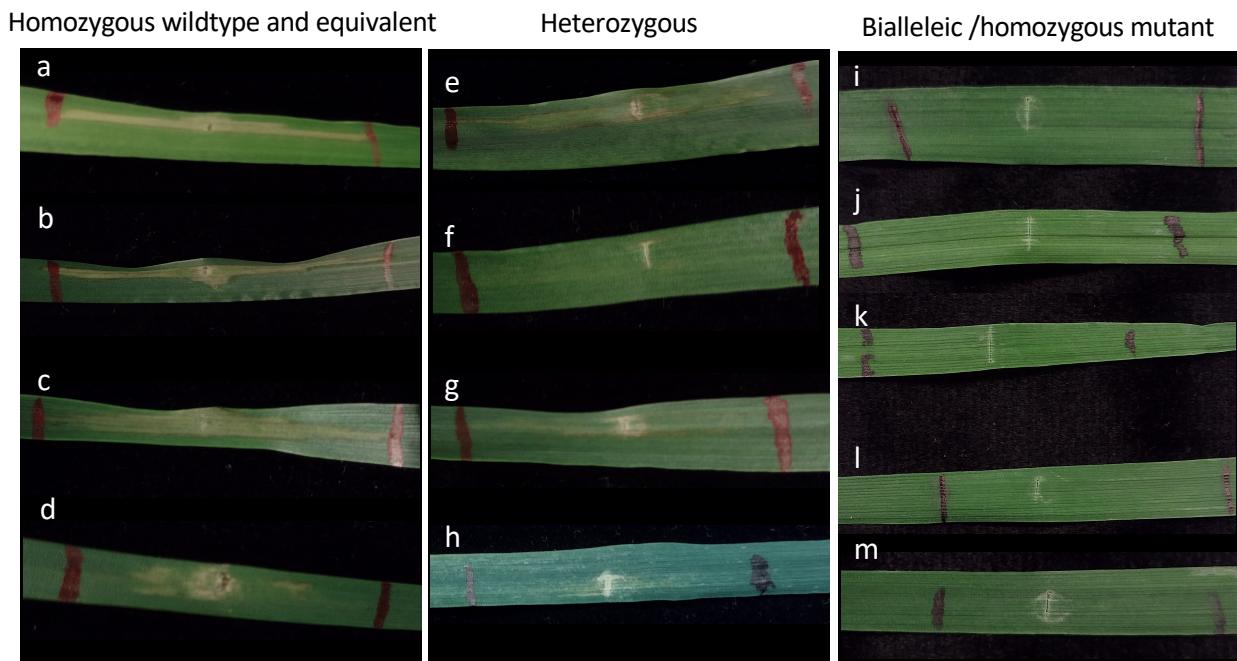
Figure 3



**Figure 4**



**Figure 5**



**Figure 6**