

Efficient CRISPR/Cas-mediated Targeted Mutagenesis in Spring and Winter Wheat Varieties

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Abstract

The CRISPR/Cas technology has recently become a molecular tool of choice for gene function studies in plants as well as crop improvement. Wheat is a globally important staple crop with a well annotated genome and there is plenty of scope for improving its agriculturally important traits using genome editing technologies, such as CRISPR/Cas. As part of this study we targeted three different genes in hexaploid wheat *Triticum aestivum*: *TaBAK1-2* in the spring cultivar Cadenza as well as *Ta-eIF4E* and *Ta-eIF(iso)4E* in winter cultivars Cezanne, Goncourt and Prevert. The primary transgenic lines carrying CRISPR/Cas-induced indels were successfully generated for all targeted genes. As winter wheat varieties are generally less amenable to genetic transformation, the successful experimental methodology for transformation and genome editing in winter wheat presented in this study will be of interest to the research community working with this crop.

Introduction

Common wheat (*Triticum aestivum*) is one of the most important staple food crops in the world. The challenges that global agriculture currently faces, such as growth of the world's population and climate change, dictate demand for technologies with a potential to accelerate crop breeding (Reynolds et al., 2021). During the last decade, genome editing emerged as a powerful new plant breeding technique (NBT) (Holme et al., 2019) that enables targeted changes in crop genomes.

CRISPR/Cas is by far the most common plant genome editing technology nowadays due to its versatility and ease of use (Kumar et al., 2021). Bread wheat is an allohexaploid, therefore it is important to have an efficient CRISPR/Cas setup as in the majority of cases, for each particular gene, one needs to target six copies i.e. two per each of the three subgenomes (A, B and D).

As part of this study, we used CRISPR/Cas in a reverse genetics approach to target the *TaBAK1-2* gene, a homologue of the Arabidopsis *BAK1* gene encoding the BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) – an important regulator of plant immunity and development (Yasuda et al., 2017; Nolan et

al., 2020), in the spring cultivar Cadenza. Here we successfully knocked out all three *TaBAK1-2* homoeologues in primary transgenic lines and demonstrated transmission of the CRISPR/Cas-induced mutant alleles to the next generation (T1). We anticipate the resultant homozygous mutant lines will facilitate studies on the involvement of BAK1 in immune responses in wheat.

In the second part of the study, we tested the potential of the CRISPR/Cas system in wheat for generating resistance to bymoviruses in the family Potyviridae, some of which are serious pathogens of crops. For instance, *Wheat spindle streak mosaic virus* (WSSMV) can pose a serious threat to wheat production in Europe and North America, while *Wheat yellow mosaic virus* (WYMV) – in East Asia (Jiang et al., 2020). Here, we targeted *Ta-eIF4E* and *Ta-eIF(iso)4E* genes encoding highly conserved translation initiation factors eIF4E and eIF(iso)4E, respectively, which serve as susceptibility (S) factors required for plant viruses from the *Potyviridae* family to complete their life cycle (Revers and García, 2015). An analogous genome editing based strategy has already been successfully used in Arabidopsis, cucumber and cassava (Schmitt-Keichinger, 2019). In addition, in barley, the conventional breeding strategies for generating resistance to bymoviruses *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV) are based on introducing recessive mutant alleles of the *eIF4E* gene (Kanyuka et al., 2005; Jiang et al., 2020). In this study, we generated genome edited wheat lines carrying indels in all three homoeologues of either *TaeIF4E* or *TaeIF(iso)4E*. These lines will be assessed for enhanced resistance to WSSMV in the follow-up study.

Material and Methods

Target sites:

The sgRNA target sites were chosen using the CRISPOR online tool (Concordet and Haeussler, 2018) or Geneious software. The target genes were sequenced in all wheat varieties used for transformation to ensure the presence of the chosen sgRNA target sites (Table 1) in each variety and in each subgenome.

Table 1
CRISPR/Cas targets.

<i>TaBAK1-2</i>	sgRNA1	GTCAAGTTCCCGAGTTCCAA
	sgRNA2	AACTTGGAGGGTGCTAATAT
	sgRNA3	GATCCAGTCGTTGTTTCGCG
<i>Ta-eIF4E</i>	sgRNA1	GCTCCACATTCAACTTGCT
	sgRNA2	GTTGTGCAACCAGAAGGTCC
	sgRNA3	GAAGGTGTGGATGGGGTGGA
	sgRNA4	GATGGTCCATTTACCGCCAT
	sgRNA5	GAAGGAGTTTCTGGACTACA
<i>Ta-eIF(iso)4E</i>	sgRNA1	GAACTCTTCGACGGTGTCGA
	sgRNA2	GGCTGGGGTAGAACCAAAGT
	sgRNA3	GACAGGATAAGCTTTCATTA
	sgRNA4	GGTCTGGATGTCGTACCAGA
	sgRNA5	GGTCGAAGCTGCGCTCCCGG

Plasmid construction

TaBAK1-2:

Three guides (guides 1, 2 and 3; Figures S1-S3) targeting *TaBAK1-2* were delivered on separate constructs, not as a multiplex array. Each guide was placed under the rice U6 promoter by cloning into the pUC19_rice_sgRNA_v2 vector (kindly provided by Keith Edwards, U. of Bristol) using BtgZI as previously described for pENTR4-sgRNA4 (Zhou et al., 2014). All three sgRNA plasmids were co-delivered along with pCas9-GFP (Zhang *et al.*, 2019) encoding the wheat codon-optimised Cas9, and pRRes1.111 (Alotaibi et al., 2018) encoding the *bar* selectable marker into immature wheat embryos (cv. Cadenza) as described below.

Ta-eIF4E/Ta-eIF(iso)4E:

To express five sgRNAs per target gene, we used sgRNA-tRNA-arrays which were constructed using a modified cloning strategy based on the report by Xie et al. (2015). In all cases, we used the Gly-tRNA sequence and an improved sgRNA backbone (Dang et al., 2015).

To target *Ta-eIF4E*, six PCRs were performed using Q5 proof-reading polymerase (NEB) with vector pUC57-R504 (kindly provided by Alison Huttly, Rothamsted Research) as template and primer pairs FH187/FH188, FH189/190, FH191/192, FH193/194, FH195/196, FH197/198. Gel-extracted PCR products were assembled in a cut-ligation reaction via BsaI-HFv2 (New England Biolabs) into vector pRRES208.482 (kindly provided by Alison Huttly, Rothamsted Research) for expression under the OsU3 promoter using previously described reaction conditions (Hahn et al., 2020), resulting in vector pFH11 (Figure S4).

Similarly, to target *Ta-eIF(iso)4E*, six PCRs were performed on vector pUC57-R504 with primer pairs FH187/FH199, FH200/FH201, FH202/FH203, FH204/FH205, FH206/FH207, FH208/FH198 and the PCR amplicons were cut-ligated into pRRES208.482 via BsaI-HFv2, resulting in vector pFH12 (Figure S5).

pFH11 was combined with pFH23 (Hahn et al., 2020), encoding wheat codon-optimised Cas9 placed under the maize ubiquitin promoter (ZmUbiPr::SpCas9), and pRRes1.111 (Alotaibi et al., 2018) encoding the *bar* selectable marker. All three plasmids were co-delivered into immature wheat embryos (cvs Cezanne, Goncourt and Prevert) as described below.

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Growth of donor plants:

The following bread wheat varieties were used for transformation: Cadenza (spring), Cezanne (winter), Goncourt (winter) and Prevert (winter).

Plants of each variety were grown in controlled environment rooms at 18°C/15°C day/night temperatures and ~700 μ M PAR for a 16 hour photoperiod. The winter varieties were initially given an 8 week vernalisation phase at 4-5°C with ~150 μ M PAR for an 8 hour photoperiod.

Transformation:

Wheat embryos of all varieties were transformed via particle bombardment essentially as previously described (Sparks and Doherty, 2020).

Donor plants were grown as above for 10-12 weeks to provide immature embryos which were isolated at 12-16 days post anthesis (dpa). The shoot/root axis was removed and the immature

scutella were plated ~ 30 per plate on the induction medium (Sparks and Doherty, 2020), and used as target tissue, giving one day pre-culture at 22°C, dark, prior to bombardment.

0.6µm gold particles (BioRad Laboratories Ltd, UK) were coated with plasmid DNAs as specified below and co-bombarded into tissues of the relevant wheat varieties using a rupture pressure of 650psi and 28.5" Hg vacuum. Following bombardment, the embryos were cultured and selected using glufosinate ammonium and putative transgenic plantlets were transferred to glasshouse conditions (all according to Sparks and Doherty, 2020).

In the case of *TaBAK1-2*, tissue culture regenerated plants were screened for the presence of transforming plasmids using the following PCR primers (see Table 2): UbiPro4 + WheatCas9R1 to test for pCas9-GFP, M13F + M13R – for sgRNA plasmids (one or more) and Bar1 + Bar2 – for pRRes1.111.

In the case of *Ta-eIF4E* and *Ta-eIF(iso)4E*, tissue culture regenerated plants were screened for the presence of transforming plasmids using the following PCR primers (see Table 2): UbiPro4 + FH147 to test for pFH23, FH209 + FH168 – for pFH11, FH209 + FH210 – for pFH12 and Bar1 + Bar2 – for pRRes1.111.

All plants generated from experiments were screened for CRISPR/Cas-induced indels using the PCR band shift assay (Nekrasov et al., 2017), whether PCR positive or negative for plasmids used for transformation.

Table 2

Primers used for genotyping of transgenic lines.

Primer	5'-3' Sequence
UbiPro4	TTTAGCCCTGCCTTCATACG
WheatCas9R1	GGATTAAGGTCGCCTTCGATGAGG
M13F	GTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGAC
Bar1	GTCTGCACCATCGTCAACC
Bar2	GAAGTCCAGCTGCCAGAAAC
FH41	GAGATTTCTGCTGAGATCCTACAT
FH42	GGATTATTACCAAACTGTGACAAT
FH43	CTGAAAGAGAAGAAGGTGGAGAT
FH44	GCTATCTGTCGGTCATCATGTAC
FH46	TCAAACGGTGTAGCGTTCTT
FH56	GTATGATCAGATTTCCGTCCG
FH57	TGAACAGCTTCGTTACTGGCAG
FH59	TGTCCAAAAGCAATCCGACC
FH147	GATGCGGAAGGTCAGGATCT
FH168	AGCAAGTTGAATGTGGGAGC
FH187	GTGGTCTCCGGCAACAAAGCACCAGTGGTCT
FH188	TAGGTCTCAATGTGGGAGCTGCACCAGCCGGAATC
FH189	GTGGTCTCCACATTCACTTGCTGTTTCAGAGCTATGCTGGGAACA
FH190	TAGGTCTCAGTTCGACAACCTGCACCAGCCGGAATC
FH191	GTGGTCTCCGAACCAAGGTCGGTTTCAGAGCTATGCTGGGAACA
FH192	TAGGTCTCACCACACCTTCTGCACCAGCCGGAATC
FH193	GTGGTCTCCGTGGATGGGGTGGAGTTTCAGAGCTATGCTGGGAACA

FH194	TAGGTCTCAATGGACCATCTGCACCAGCCGGAATC
FH195	GTGGTCTCCCCATTTACCGCCATGTTTCAGAGCTATGCTGGGAACA
FH196	TAGGTCTCAAACCTCTTCTGCACCAGCCGGAATC
FH197	GTGGTCTCCAGTTTCTGGACTACAGTTTCAGAGCTATGCTGGGAACA
FH198	GTGCGTCTCCAAACAAAAAAGCACCGACTCGGTGCCACT
FH199	TAGGTCTCACGAAGAGTTCTGCACCAGCCGGAATC
FH200	GTGGTCTCCTTCGACGGTGTCTGAGTTTCAGAGCTATGCTGGGAACA
FH201	TAGGTCTCATACCCAGCCTGCACCAGCCGGAATC
FH202	GTGGTCTCCGGTAGAACCAAGTGTTCAGAGCTATGCTGGGAACA
FH203	TAGGTCTCATTATCCTGTCTGCACCAGCCGGAATC
FH204	GTGGTCTCCATAAGCTTTCATTAGTTTCAGAGCTATGCTGGGAACA
FH205	TAGGTCTCACATCCAGACCTGCACCAGCCGGAATC
FH206	GTGGTCTCCGATGTCGTACCAGAGTTTCAGAGCTATGCTGGGAACA
FH207	TAGGTCTCAGCTTCGACCTGCACCAGCCGGAATC
FH208	GTGGTCTCCAAGCTGCGCTCCCGGTTTCAGAGCTATGCTGGGAACA
FH209	TGAAGCCTTTCAGGACATGTAT
FH210	CACTATAGGGCGAATTGGAGA
FH221	TAGAAAAGCCCAGCTTTC
FH224	ATTAGCTGAGGCGTTTCAATCT
FH227	TGTAACACGACGGCCAGTTTGGCGCTCTCATTATGTAT
FH228	CCTCTCTATGGGCAGTCGGTGATAGGAATCGCAACTTTAAGAGTT
FH230	CCTCTCTATGGGCAGTCGGTGATCATGCCACAGCATATCCT
FH431	TCCCCATCCCAAACCTCG
FH445	TGTTACCAATCATTGCCAGC
FH447	ATTTACCAGATAAGCATTGGCAA

Analysis of CRISPR/Cas-induced mutations:

TaBAK1-2:

Similarly, primary (T0) transformants were analysed for mutations in the *TaBAK1-2* gene using the PCR band shift assay with primers FH41/FH44 (amplifying across all three sgRNA targets), FH41/FH42 (amplifying across sgRNA1 and 2 targets) and primers FH43/FH44 (amplifying across the sgRNA3 target). If amplicon band shifts were visible, target genes were amplified again using Q5 DNA Polymerase (New England Biolabs) with the same primer pairs as before. The PCR products were sub-cloned using the Zero Blunt™ TOPO™ PCR Cloning Kit (Thermo Fisher Scientific) and multiple single clones were Sanger-sequenced (Eurofins Genomics) to detect and analyse mutations in all subgenomes.

In the case of *TaBAK1-2*, allele profiling was performed by PCR for T1 progeny lines derived from one of the T0 transformants. This was possible because each of the *TaBAK1-2* alleles from all three subgenomes carried distinct indels that resulted in clearly distinguishable migration patterns of PCR products.

Ta-eIF4E and *Ta-eIF(iso)4E*:

Primary (T0) transformants were initially screened for CRISPR/Cas-induced mutations in the *Ta-eIF4E* and *Ta-eIF(iso)4E* genes using the PCR band shift assay. For this, the target genes were amplified using DreamTaq DNA polymerase (ThermoFisher Scientific) and primers FH46 + FH221 (full gene), FH445 + FH221 (5' part of the gene) and FH447 + FH46 (3' part of the gene) (Table 2) in plants

transformed with the vector pFH11 (in the case of targeting *Ta-eIF4E*) or primers FH431 + FH224 (full gene), FH431 + FH59 (5' part of gene) and FH56 + FH57 (3' part of gene) (Table 2) in plants transformed with the vector pFH12 (in the case of targeting *Ta-eIF(iso)4E*). If band shifts were detected, the respective gene fragments were amplified using Q5® DNA Polymerase and subcloned. Multiple single clones were Sanger-sequenced, as described above for *TaBAK1-2*.

Results

Targeted mutagenesis of the *TaBAK1-2* gene

We used the CRISPR/Cas system to target three *TaBAK1-2* homoeologues located on chromosome 2: *TraesCS2A02G343100* (*TaBAK1-2A*), *TraesCS2B01G340700* (*TaBAK1-2B*) and *TraesCS2D02G321400* (*TaBAK1-2D*). All three homoeologues were targeted at three conserved sgRNA target sites within exons 4 (sgRNA1), 5 (sgRNA2) and 11 (sgRNA3) (Figure 1A). We transformed wheat immature embryos (cv Cadenza) with DNA constructs expressing CRISPR/Cas reagents, as described in Materials and Methods, and regenerated 30 T0 primary transgenic lines. We then genotyped the T0 lines for the presence of CRISPR/Cas-induced indels using the PCR band shift assay (Nekrasov et al., 2017). Out of the 30 T0 lines, two showed a clear PCR band shift indicating presence of deletions of around 600 bp (Figure 1B). To identify *TaBAK1-2* alleles present in both T0 plants, we subcloned the PCR amplicons into a high copy number vector and sequenced individual clones by Sanger. The T0 plant 1 turned out to be a triple-biallelic carrying indels in all six copies of *TaBAK1*, while the plant 2 carried heterozygous mutations in *TaBAK1-2A* and *TaBAK1-2D* and no mutations in *TaBAK1-2B* (Figure 1C and D). The three insertions identified in the T0 plant 1 (43 bp, 81 bp and 136 bp; Figure 1C) proved to be fragments of the pCas9-GFP plasmid. Such insertion events at CRISPR/Cas target sites were previously reported in potato (Andersson et al., 2017, 2018).

It should be noted that PCR amplicons shown in Figure 1B were produced using primers amplifying across the sgRNA1 and 2 target sites. We also separately amplified across the sgRNA3 target in both T0 plants and did not detect any mutations at this site.

We selected the *tabak1-2* T0 line 1 for further analysis as this line showed mutations in all six copies of the *TaBAK1-2* gene.

To check if the mutations present in the *tabak1-2* T0 line 1 could be transmitted through the germline and inherited by the next generation, we PCR-genotyped 52 T1 progeny plants derived from this line. The genotyping data clearly indicated inheritance of all six mutant alleles (Figures 2, S6 and S7). Out of 52 T1 lines, five were triple-homozygous (plants 4, 19, 23, 43 and 52; Figure S7).

Targeted mutagenesis of the *Ta-eIF4E* and *Ta-eIF(iso)4E* genes

We targeted both *Ta-eIF4E* (homoeologues *TraesCS3A02G521500*, *TraesCS3B02G591300* and *TraesCS3D02G527800*) and *Ta-eIF(iso)4E* (homoeologues *TraesCS1A02G149200*, *TraesCS1B02G167100* and *TraesCS1D02G146500*) genes with five sgRNAs each (Figures 3A and 4A, respectively) in three winter cultivars of common wheat, Cezanne, Goncourt and Prevert, which are susceptible to WSSMV (Dragan Perovic, personal communication).

In total, we screened 49 T0 plants for *Ta-eIF4E* (40, 8 and 1 from cvs Cezanne, Goncourt and Prevert transformations, respectively) and 40 T0 plants for *Ta-eIF(iso)4E* (30, 5 and 5 from cvs Cezanne, Goncourt and Prevert transformations, respectively). Genotyping identified two T0 plants carrying CRISPR/Cas-induced indels in *Ta-eIF4E* (cvs Cezanne and Goncourt; Figures 3B and S8) and two T0

plants with indels in *Ta-eIF(iso)4E* (cvs Cezanne and Prevert; Figures 4B, S9 and S10). Two out of the four T0 plants were triple-biallelic: *ta-eif4e* T0 plant 1 (cv Cezanne; Figures 3B and S8) and *ta-eif(iso)4e* T0 plant 2 (cv Prevert; Figures 4B and S10).

As in the case of *TaBAK1-2*, we detected CRISPR/Cas-induced insertions in *ta-eif4e* and *ta-eif(iso)4e* T0 lines ranging from 242 bp to 592 bp (Figures S8-S10). The inserted DNA was derived from plasmids used for transformation, wheat genomic DNA, bacterial DNA or combinations of those.

Discussion

During this study we generated *tabak1-2* lines carrying different combinations of mutant *tabak1-2a*, *tabak1-2b* and *tabak1-2d* alleles (Figure S7). BAK1 acts as a coreceptor for a number of pattern recognition receptors (PRRs) involved in pattern-triggered immunity (PTI) responses in plants (Yasuda et al., 2017). The generated *tabak1-2* alleles are expected to be loss-of-function as they carry rather large deletions/insertions within the coding regions or short deletions (e.g. -4 bp) that put the coding sequence out of frame. We therefore expect the *tabak1-2* lines to become a useful genetic resource for the research community working on molecular mechanisms of plant-microbe interactions in wheat.

To generate the *ta-eif4e* and *ta-eif(iso)4e* lines, we needed to optimise the transformation procedure for the three winter wheat cultivars Cezanne, Goncourt and Prevert (see Materials and Methods). Since there are only few published examples of genome editing in winter wheat (Li et al., 2020; Liu et al., 2021), our work will be of interest to researchers working on winter wheat transformation and genome editing.

To be able to evaluate *ta-eif4e* and *ta-eif(iso)4e* mutants for enhanced resistance to WSSMV, phenotypic characterisation of these lines would need to be carried out in a follow-up study. Since WSSMV is transmitted by *Polymyxa graminis* (Jiang et al., 2020), a soil-borne filamentous microorganism infecting wheat roots, it is very difficult to perform WSSMV pathotests under the glasshouse conditions. On the other hand, GM field trials in the south of Europe, where WSSMV can be found, are problematic right now due to the policy restrictions and significant public opposition. We are hopeful the situation will change at some point in the future. It should be noted, that since some of the *ta-eif4e* and *ta-eif(iso)4e* mutant alleles contain inserted fragments of transgenic or wheat genomic DNA in them, plants carrying them cannot be treated as transgene-free genome edited but rather GM lines.

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Figure legends

Figure 1.

Targeted mutagenesis of *TaBak1-2*. (A) Cartoon showing locations of sgRNA target sites. (B) PCR genotyping of CRISPR/Cas-mutagenised T0 lines. (C) Mutant *tabak1-2* alleles identified by Sanger sequencing in T0 plants. (D) Allele composition of *tabak1-2* T0 plants.

Figure 2.

CRISPR/Cas-induced *tabak1-2* mutant alleles are inherited by the next generation. The table shows allele distribution among T1 progeny of the T0 plant 1.

Figure 3.

Targeted mutagenesis of *Ta-eIF4e*. (A) Cartoon showing locations of sgRNA target sites. (B) Allele composition of *ta-eif4e* T0 plants.

Figure 4.

Targeted mutagenesis of *Ta-eIF(iso)4e*. (A) Cartoon showing locations of sgRNA target sites. (B) Allele composition of *ta-eif(iso)4e* T0 plants.

References

- Alotaibi, S. S., Sparks, C. A., Parry, M. A. J., Simkin, A. J., and Raines, C. A. (2018). Identification of leaf promoters for use in transgenic wheat. *Plants* 7, 27. doi: 10.3390/plants7020027.
- Andersson, M., Turesson, H., Nicolai, A., Falt, A. S., Samuelsson, M., and Hofvander, P. (2017). Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Reports* 36, 117–128. doi: 10.1007/s00299-016-2062-3.
- Andersson, M., Turesson, H., Olsson, N., Fält, A.-S., Ohlsson, P., Gonzalez, M. N., et al. (2018). Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum* 164, 378–384. doi:10.1111/ppl.12731.
- Concordet, J.-P., and Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Research* 46, W242–W245. doi: 10.1093/nar/gky354.
- Dang, Y., Jia, G., Choi, J., Ma, H., Anaya, E., Ye, C., et al. (2015). Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. *Genome Biology* 16, 280. doi: 10.1186/s13059-015-0846-3.
- Hahn, F., Korolev, A., Sanjurjo Loures, L., and Nekrasov, V. (2020). A modular cloning toolkit for genome editing in plants. *BMC Plant Biology* 20, 179. doi: 10.1186/s12870-020-02388-2.
- Holme, I. B., Gregersen, P. L., and Brinch-Pedersen, H. (2019). Induced genetic variation in crop plants by random or targeted mutagenesis: convergence and differences. *Frontiers in Plant Science* 10, 1468. doi: 10.3389/fpls.2019.01468.

- Jiang, C., Kan, J., Ordon, F., Perovic, D., and Yang, P. (2020). Bymovirus-induced yellow mosaic diseases in barley and wheat: viruses, genetic resistances and functional aspects. *Theoretical & Applied Genetics* 133, 1623–1640. doi: 10.1007/s00122-020-03555-7.
- Kanyuka, K., Druka, A., Caldwell, D. G., Tymon, A., McCallum, N., Waugh, R., et al. (2005). Evidence that the recessive bymovirus resistance locus *rym4* in barley corresponds to the *eukaryotic translation initiation factor 4E* gene. *Molecular Plant Pathology* 6, 449–458. doi: <https://doi.org/10.1111/j.1364-3703.2005.00294.x>.
- Kumar, S., Rymarquis, L. A., Ezura, H., and Nekrasov, V. (2021). Editorial: CRISPR-Cas in agriculture: opportunities and challenges. *Frontiers in Plant Science* 12, 672329. doi: 10.3389/fpls.2021.672329.
- Li, J., Jiao, G., Sun, Y., Chen, J., Zhong, Y., Yan, L., et al. (2020). Modification of starch composition, structure and properties through editing of *TaSBEIIa* in both winter and spring wheat varieties by CRISPR/Cas9. *Plant Biotechnology Journal*. doi: 10.1111/pbi.13519.
- Liu, Y., Luo, W., Linghu, Q., Abe, F., Hisano, H., Sato, K., et al. (2021). *In planta* genome editing in commercial wheat varieties. *Frontiers in Plant Science* 12, 648841. doi: 10.3389/fpls.2021.648841.
- Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D., and Kamoun, S. (2017). Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports* 7, 482. doi: 10.1038/s41598-017-00578-x.
- Nolan, T. M., Vukašinović, N., Liu, D., Russinova, E., and Yin, Y. (2020). Brassinosteroids: multidimensional regulators of plant growth, development, and stress responses. *The Plant Cell* 32, 295–318. doi: 10.1105/tpc.19.00335.
- Revers, F., and García, J. A. (2015). “Chapter Three - Molecular Biology of Potyviruses,” in *Advances in Virus Research*, eds. K. Maramorosch and T. C. Mettenleiter (Academic Press), 101–199. doi: 10.1016/bs.aivir.2014.11.006.
- Reynolds, M., Atkin, O. K., Bennett, M., Cooper, M., Dodd, I. C., Foulkes, M. J., et al. (2021). Addressing research bottlenecks to crop productivity. *Trends in Plant Science*. doi: 10.1016/j.tplants.2021.03.011.
- Schmitt-Keichinger, C. (2019). Manipulating cellular factors to combat viruses: a case study from the plant eukaryotic translation initiation factors eIF4. *Frontiers in Microbiology* 10, 17. doi: 10.3389/fmicb.2019.00017.
- Sparks, C. A., and Doherty, A. (2020). “Genetic Transformation of Common Wheat (*Triticum aestivum* L.) Using Biolistics,” in *Biolistic DNA Delivery in Plants: Methods and Protocols* Methods in Molecular Biology., eds. S. Rustgi and H. Luo (New York, NY: Springer US), 229–250. doi: 10.1007/978-1-0716-0356-7_12.
- Xie, K., Minkenberg, B., and Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of the United States of America* 112, 3570–3575. doi: 10.1073/pnas.1420294112.
- Yasuda, S., Okada, K., and Saijo, Y. (2017). A look at plant immunity through the window of the multitasking coreceptor BAK1. *Current Opinion in Plant Biology* 38, 10–18. doi: 10.1016/j.pbi.2017.04.007.

Zhou, H., Liu, B., Weeks, D. P., Spalding, M. H., and Yang, B. (2014). Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Research* 42, 10903–10914. doi: 10.1093/nar/gku806.

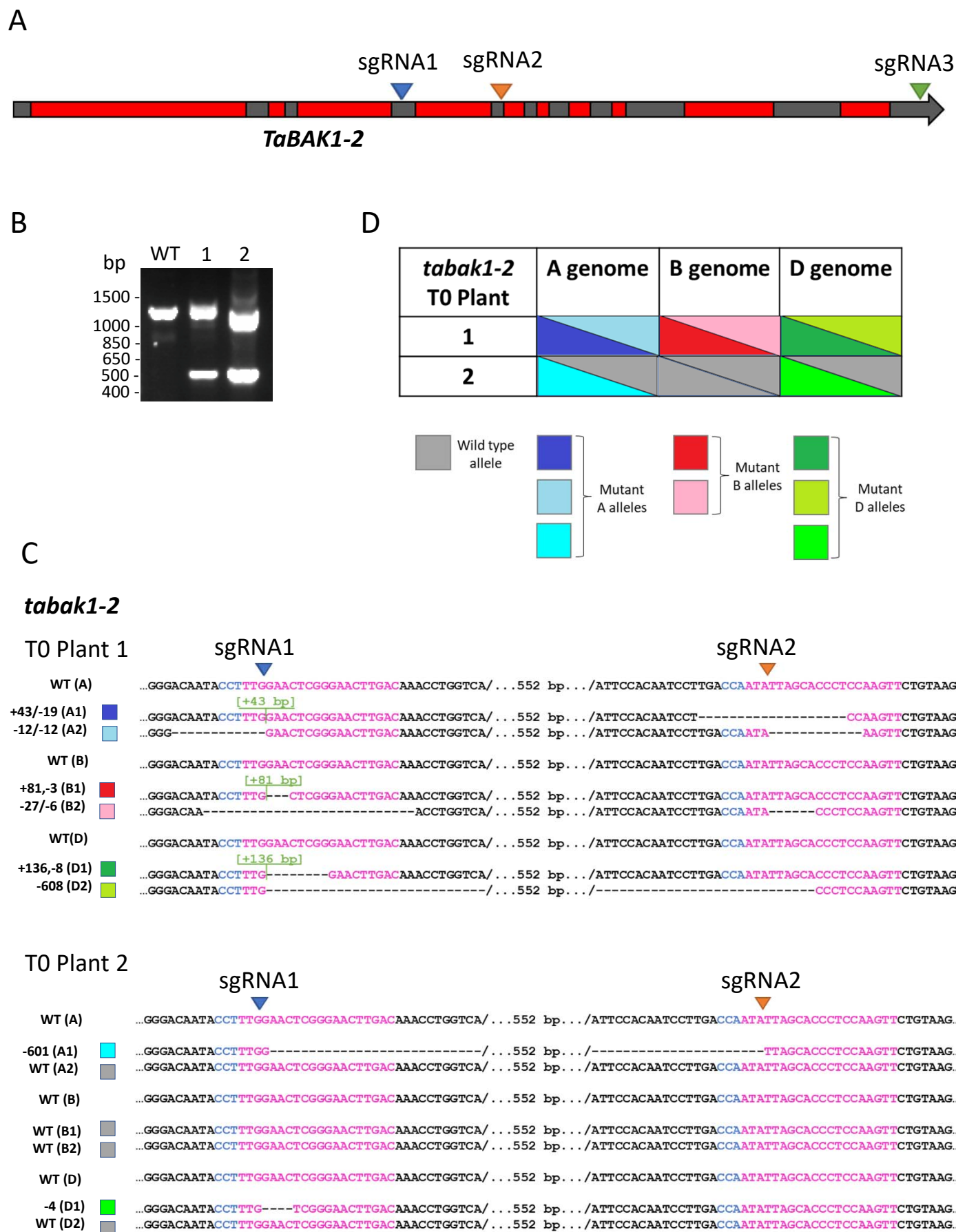


Figure 1



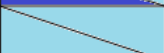






	Genotype	Number of T1 plants
A genome		17
		26
		8
B genome		7
		34
		10
D genome		8
		29
		14

Figure 2

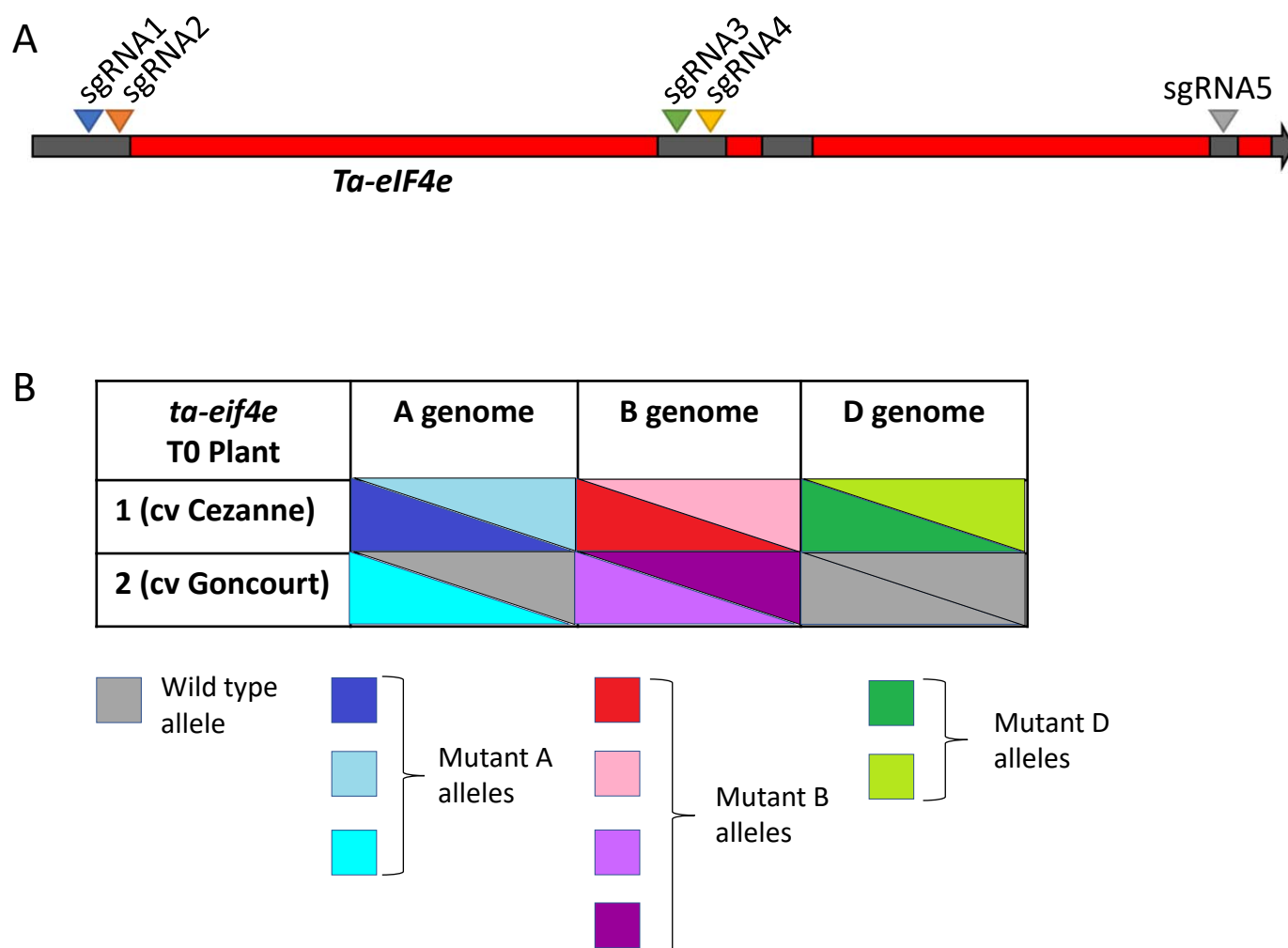
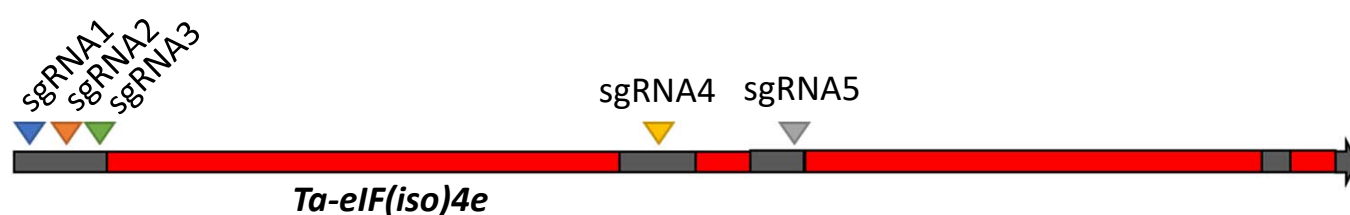


Figure 3

A



B

<i>ta-eif(iso)4e</i> T0 Plant	A genome	B genome	D genome
1 (cv Cezanne)			
2 (cv Prevert)			

Wild type allele

Mutant A alleles

Mutant B alleles

Mutant D alleles

Figure 4