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Highly efficient heritable genome editing in wheat using an RNA virus and bypassing tissue culture

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ABSTRACT

Genome editing provides novel strategies for improving plant traits but mostly relies on conventional plant genetic transformation and regeneration procedures, which can be inefficient. In this study, we have engineered a *Barley stripe mosaic virus*–based sgRNA delivery vector (BSMV-sg) that is effective in performing heritable genome editing in Cas9-transgenic wheat plants. Mutated progenies were present in the next generation at frequencies ranging from 12.9% to 100% in three different wheat varieties, and 53.8%–100% of mutants were virus free. We also achieved multiplex mutagenesis in progeny using a pool of BSMV-sg vectors harboring different sgRNAs. Furthermore, we devised a virus-induced transgene-free editing procedure to generate Cas9-free wheat mutants by crossing BSMV-infected Cas9-transgenic wheat pollen with wild-type wheat. Our study provides a robust, convenient, and tissue culture–free approach for genome editing in wheat through virus infection.

Key words: heritable genome editing, BSMV, common wheat, tissue culture-free, multiplex editing

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INTRODUCTION

Efficient delivery systems are needed for high-throughput genome editing in plants (Atkins and Voytas, 2020; Gao, 2021). Current plant genome editing is typically conducted by delivering reagents, such as Cas9 and single guide RNAs (sgRNAs), by conventional *Agrobacterium*-mediated gene delivery and particle bombardment (Altpeter et al., 2016; Ran et al., 2017; Chen et al., 2019). However, these systems are limited to a narrow range of genotypes due to a restricted ability to regenerate plants. In addition, almost all the current methods require tissue culture, a time-consuming and laborious process that impedes routine application of genome editing in both dicots and monocots (Ran et al., 2017; Gao, 2021). Although some developmental regulators, such as the *WUSCHEL*, *BABY BOOM*, and *GROWTH-REGULATING FACTOR* families, have been developed to improve plant regeneration, they also tend to affect the normal development of the regenerated plants and still require tissue culture (Lowe et al., 2016; Debernardi et al., 2020; Kong et al., 2020). Therefore, a new system is needed that delivers CRISPR system components into plant germline or meristematic cells and achieves genotype-independent editing without requiring tissue culture.

Plant viruses have been manipulated to express foreign proteins and specific segments of RNA in a wide range of plant hosts (Wang et al., 2016, 2020; Zhao et al., 2016; Cody and Scholthof, 2019; Peng et al., 2020; Oh et al., 2021). In particular, *Agrobacterium*-based viral vectors have been developed that load these viruses into plant cells by simple agroinfiltration methods (Annamalai and Rao, 2005; Cody and Scholthof, 2019). Based on this, several plant RNA virus–based vector systems can now deliver genome editing reagents into plant leaves (Ali et al., 2015, 2018; Cody et al., 2017; Kaya et al., 2017; Gao et al., 2019; Hu et al., 2019; Jiang et al., 2019; Mei et al., 2019; Ma et al., 2020; Zhang et al., 2020). A *Tobacco rattle virus* (TRV)-based heritable gene editing system was

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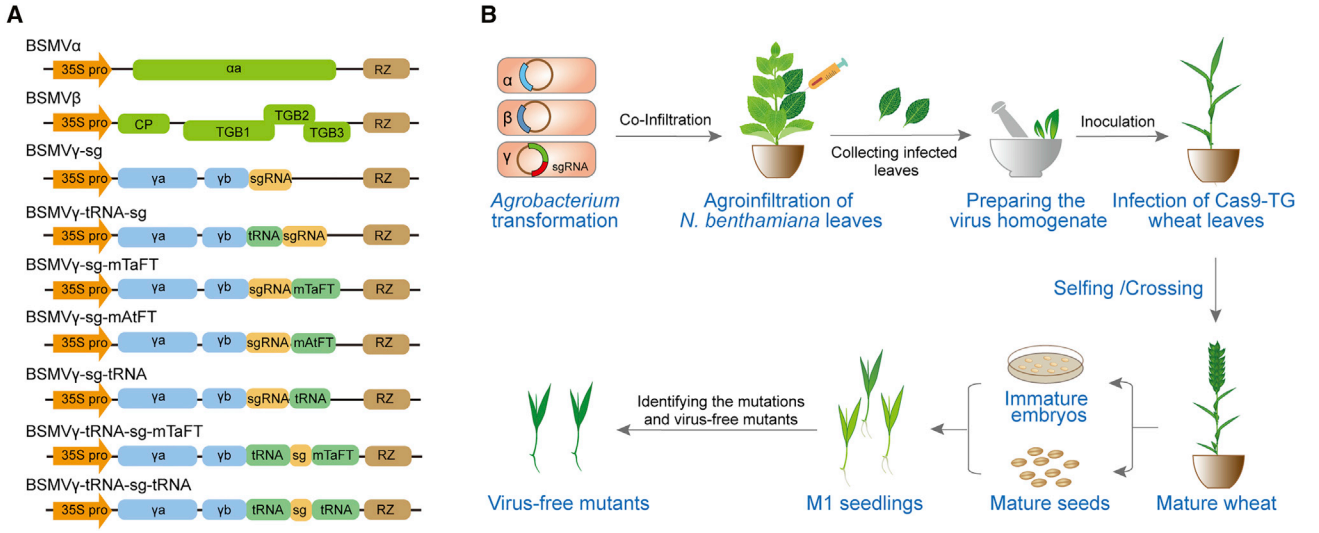


Figure 1. Schematic representation of engineered BSMV vectors carrying different sgRNAs for genome editing in wheat.
(A) Schematic representation of the BSMV-sg system, including BSMVα, BSMVβ, and the seven engineered BSMVγ-sg vectors.
(B) Overview of BSMV-sg-mediated heritable genome editing in wheat. BSMV-sg vectors were transformed into *Agrobacterium* cells and co-infiltrated into *N. benthamiana* leaves; at 3–7 days post-inoculation, inoculated leaves were collected and homogenized in a mortar containing an inoculation buffer, and the homogenates were rub-inoculated onto the leaves of Cas9-transgenic (Cas9-TG) wheat at the jointing stage. The M1 generation was obtained either by planting harvested seeds or by rescuing immature embryos of the infected M0 wheat plants. Mutated progenies among the M1 seedlings were screened by PCR restriction-enzyme digestion assays and NGS analysis, and virus-free mutants were identified by RT-PCR.

established in Cas9-transgenic *Nicotiana benthamiana* by fusing a mobile RNA element (*Flowering Locus T*, *FT*) to the 3′ end of the sgRNA, which may help the guide RNA enter meristems to produce heritable changes (Ellison et al., 2020). A *Potato virus X* (PVX) vector has also been engineered using a similar strategy to express sgRNA arrays for multiplex genome editing in *N. benthamiana*, and virus-free mutated progenies can be obtained from infected plant seeds (Uranga et al., 2021). In addition, due to the large capacity of rhabdoviruses, a sonchus yellow net rhabdovirus vector has been generated that delivers an entire CRISPR-Cas9 cassette and achieves DNA-free genome editing in *N. benthamiana*, although it still relies on tissue culture to obtain mutant seedlings (Ma et al., 2020). However, due to host range restriction, all of these RNA virus–mediated genome editing tools can mainly be used in dicotyledonous model plants, such as *N. benthamiana*. So far, no virus-mediated heritable gene editing tool is available for monocots, in particular for hexaploid wheat (*Triticum aestivum*).

Barley stripe mosaic virus (BSMV) is a positive-strand RNA virus with a tripartite genome designated RNA α, β, and γ, which was developed as a virus-induced gene-silencing vector for high-throughput genomics studies in plants (Holzberg et al., 2002; Yuan et al., 2011; Bennypaul et al., 2012; Ma et al., 2012). It has been engineered for sgRNA delivery to edit host genes in the leaves of Cas9-transgenic *N. benthamiana*, wheat, and maize (Hu et al., 2019), but its ability to perform heritable editing remains to be explored. Here, we developed a BSMV-mediated sgRNA (BSMV-sg) delivery system for different wheat varieties, and found that it was effective in editing a variety of Cas9-transgenic wheat varieties, without requiring tissue culture and regeneration. Using this system, we obtained wheat seedlings harboring desired mutations in up to 100% of the M1 generation. Homozygous mutants with all six alleles simultaneously edited were also present in the M1 generation. Moreover, 53.8%–100% of the M1 progeny mutants were virus free and their edits were transmitted to the M2 generation. We also obtained multiplex-edited mutants using a pool of BSMV-sgs harboring different sgRNAs. Furthermore, Cas9-free derivatives could be produced by crossing infected Cas9 plants with wild-type wheat.

RESULTS

Highly efficient somatic genome editing mediated by the BSMV-sg system in wheat

To develop BSMV-mediated heritable editing in wheat, we first integrated an sgRNA downstream of the γb open reading frame in RNAγ, to produce BSMVγ-sg, and co-expressed it with BSMVα and BSMVβ to produce the BSMV-sg system (Figure 1A). Recently, several studies have reported that the introduction of mobile RNA elements, such as tRNA (Zhang et al., 2016a) and mutated AtFT (mAtFT) (Li et al., 2009), into the viral vector can promote movement of sgRNA transcripts into shoot apical meristem cells (Ellison et al., 2020; Lei et al., 2021; Uranga et al., 2021). So, we fused several mobile RNA elements, including mAtFT, mTaFT (a truncated wheat *FT* RNA sequence, ortholog of mAtFT), and $tRNA^{Met}$, to the 5′ and/or 3′ end of the sgRNA (Supplemental Figure 1), to produce six BSMVγ-sg derivatives depicted in Figure 1A.

To test whether these BSMV-sg vectors carry out efficient genome editing in wheat, an sgRNA targeting a conserved region of the six alleles of the wheat *phytoene desaturase* (*TaPDS*) gene was selected, and the sgRNA was introduced into the seven

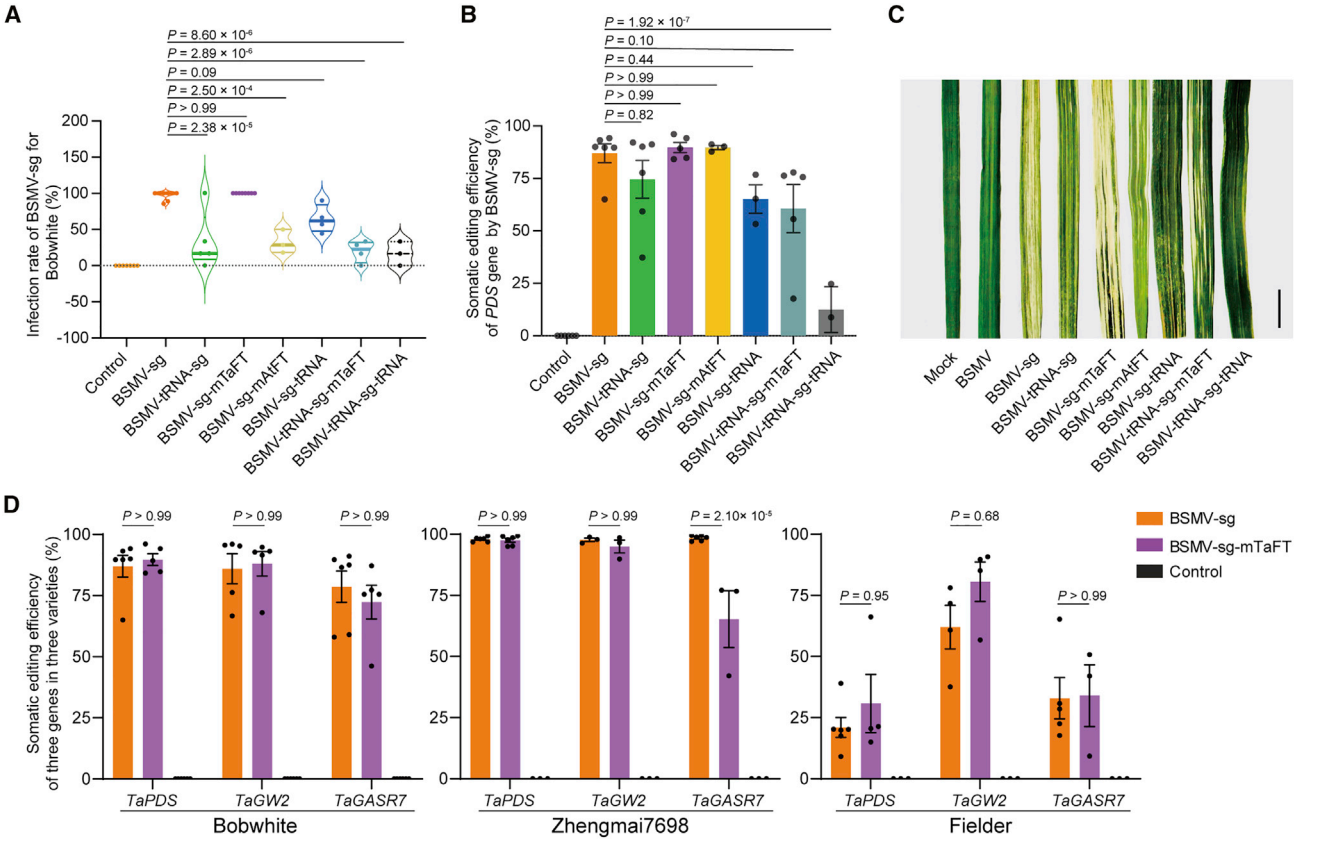


Figure 2. Efficient somatic gene editing using BSMV vectors in wheat.

(A) Comparison of viral infection rates in Bobwhite by inoculating with the seven BSMV-derived vectors. Buffer-inoculated Bobwhite served as a control. Four to eleven plants were inoculated for each replicate, and the violin plot elements show the infection efficiency distribution with medians and quartiles.

(B) Comparison of the editing efficiencies of infected Cas9-transgenic Bobwhite with the seven BSMV-sg vector systems (n ≥ 3). Buffer-inoculated Bobwhite served as control.

(C) Representative phenotypes of Cas9-transgenic Bobwhite leaves infected with the seven BSMV vectors targeting *TaPDS*. Uninfected Bobwhite leaves (Mock) and wild-type BSMV-infected leaves (BSMV) served as controls. Scale bar, 1 cm.

(D) Comparison of genome editing efficiencies of BSMV-sg and BSMV-sg-mTaFT at target sites of *TaPDS*, *TaGW2*, and *TaGASR7* in Cas9-transgenic Bobwhite, Zhengmai 7698, and Fielder. Uninoculated wheat plants served as controls.

(A, B, and D) Significances are indicated among different groups by exact *P* value except when *P* > 0.99 (ANOVA, Tukey's honest significant difference [HSD]). Data are means ± SEM.

engineered BSMVγ vectors. The procedures for infection and subsequent genome editing of wheat are shown in Figure 1B. Each of the BSMVγ vectors was transformed individually into *Agrobacterium* cells and mixed with an equal concentration of *Agrobacterium* transformed with the BSMVα and BSMVβ vectors; the mixtures were then used to agroinfiltrate *N. benthamiana* leaves to recover the BSMV derivatives (Figure 1B). Five days post-inoculation (dpi), inoculated leaves were collected and homogenized with an inoculation buffer in a mortar. The homogenates were then inoculated by rubbing the leaves of Cas9-transgenic Bobwhite wheat plants. By about 7–15 dpi, BSMV-induced symptoms characterized by chlorotic spots and stripes appeared on the upper uninoculated leaves. The viral infection rates of different BSMV vectors in wheat were calculated from at least three replicates of inoculation (4–11 plants were used for each inoculation), and we found that the original vector BSMV-sg and BSMV-sg-mTaFT vectors had higher viral infection rates (ranging from 85.7% to 100%) than the other constructs in most replicates (Figure 2A and Supplemental Table 1), indicating that the modifications to the sgRNA in the latter constructs may negatively affect viral viability.

At approximately 15 dpi, we examined targeted mutagenesis of the endogenous gene *TaPDS*. Genome DNA was extracted from upper uninoculated leaves displaying symptoms in the plants inoculated with the seven engineered BSMV constructs, and a pair of conserved primers was used to amplify the three *TaPDS* loci for sequencing (Figure 1B). Next-generation sequencing (NGS) showed that all seven viral vectors had produced targeted mutations in the systemically infected leaves, with editing efficiencies from 3.8% to 96.1%. However, BSMV-sg, BSMV-sg-mTaFT, and BSMV-sg-mAtFT yielded higher mutation frequencies than the other constructs (Figure 2B). Furthermore, some of the plants infected with the BSMV vectors carrying the various modified sgRNAs had bleached stripes on their systemic leaves; this was especially the case for the BSMV-sg and BSMV-sg-mTaFT constructs, which induced a more conspicuous albino phenotype than the others

(Figure 2C and Supplemental Figure 2). These results suggest that introduction of the mobile RNA element mTaFT into the BSMVγ-sg vector did not impair viral activity, thus holding out the prospect that heritable gene edits could be generated. We selected the original BSMV-sg and BSMV-sg-mTaFT for subsequent studies.

To investigate the versatility of the BSMV vectors, we designed another two sgRNAs, targeting *TaGW2* and *TaGASR7*, and tested these constructs in another two Cas9-transgenic wheat varieties, Fielder and Zhengmai 7698 (an elite wheat variety widely cultivated in China). NGS analysis showed that both BSMV-sg and BSMV-sg-mTaFT also efficiently edited *TaGW2* and *TaGASR7* in Bobwhite and Zhengmai 7698, with editing efficiencies ranging from 42.1% to 99.5% (Figure 2D). There was no significant difference in editing rates between BSMV-sg and BSMV-sg-mTaFT except for *TaGASR7* in Zhengmai 7698 (Figure 2D). However, BSMV-sg and BSMV-sg-mTaFT exhibited reduced editing rates, ranging from 9.1% to 90.8%, in Cas9-transgenic Fielder, which may be due to the lower expression level of Cas9 protein in Fielder (Figure 2D and Supplemental Figure 3). These results indicate that BSMV-sg and BSMV-sg-mTaFT are highly effective in generating somatic edits at different loci in different wheat varieties.

BSMV-mediated genome editing is heritable in wheat

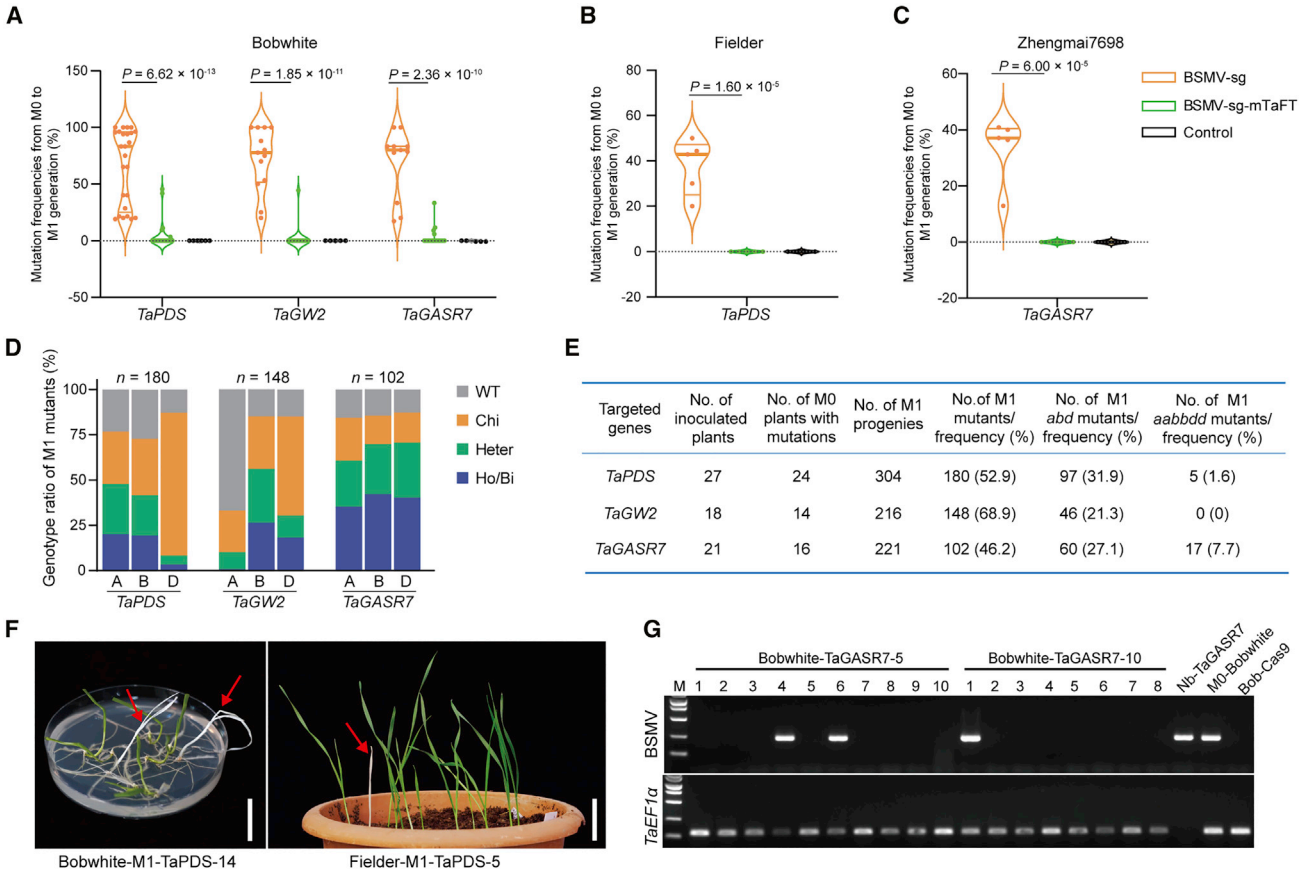
To investigate whether the mutation observed in the systemically infected leaves of wheat could be transmitted to the next generation through seeds. we first set out to identify mutations in the M1 progeny seedlings from the parental Bobwhite plants that infected by BSMVγ-sg and BSMV-sg-mTaFT for targeting *TaPDS*, *TaGW2*, and *TaGASR7* (Figure 3A). The seedlings were obtained by direct rescue of immature embryos for the sake of saving time (Figure 1B). NGS showed that M1 progeny seedlings from all BSMV-sg-infected plants contained mutations, and the mutation frequencies ranged from 20.0% to 100% in three targeted sites (Figure 3A). Surprisingly, most of the M1 seedlings from the BSMV-sg-mTaFT-infected plants failed to harbor mutations in these target sites (Figure 3A). To confirm this result, we also checked for mutations in *TaPDS* and *TaGASR7* in Cas9-transgenic Fielder and Zhengmai 7698, respectively, and found that BSMV-sg induced efficient heritable editing in these two wheat varieties, with 12.9%–50.0% of the M1 progenies containing targeted mutations; but again, no mutations were detected in the offspring when the two wheat varieties were infected with BSMV-sg-mTaFT (Figure 3B and 3C). Although studies have shown that mobile RNA elements facilitated heritable gene editing by TRV-, PVX-, and cotton leaf crumple virus (CLCrV)-based gene editing vectors in *N. benthamiana* and *Arabidopsis thaliana* (Ellison et al., 2020; Lei et al., 2021; Uranga et al., 2021), we found that fusion of *FT* RNA and/or tRNA to the sgRNA within the BSMV-sg vector failed to stimulate, and even decreased the efficiency of heritable editing. This suggests that fusion of an *FT* element to the sgRNA in BSMVγ may impair its ability to enter meristematic cells, and the original BSMV-sg could perform efficient heritable genome editing in wheat.

Next, to investigate which genomes the mutations in the M1 mutants occurred in by BSMV-sg, we designed primer pairs to amplify the *TaPDS*, *TaGW2*, and *TaGASR7* targets separately in the A/B/D subgenomes of Bobwhite. Thereafter, NGS results revealed that BSMV-sg-induced mutations were present in all three diploid genomes and that almost half of the mutant progenies were heterozygous and homozygous, and a significant portion were chimeric (having more than two genotypes at a target site or a deviated mutation frequency from 15.0% to 35.0% or from 65.1% to 85.0%) (Figure 3D and Supplemental Figure 4). For example, among the 180 *TaPDS* mutants of three wheat varieties, we identified 50 heterozygous mutants for *TaPDS-A1*, 40 heterozygous mutants for *TaPDS-B1*, 9 heterozygous mutants for *TaPDS-D1*, and 97 mutants with all three homeoalleles mutated, of which 5 were homozygous mutants with six alleles simultaneously being edited (Figure 3D and 3E). Notably, we also observed fully bleached phenotypes among the Bobwhite and Fielder M1 seedlings derived from parental plants infected with BSMV-sg targeting *TaPDS* (Figure 3F), indicating that the BSMV-sg was effective in inducing robust homozygous mutations simultaneously in the three homeoalleles of wheat. Highly efficient heritable gene editing was also detected for *TaGW2* and *TaGASR7* (Figure 3D and 3E). All the heterozygous and homozygous mutations could be transmitted to the M2 generation from the tested M1 mutants (Supplemental Tables 2, 3 and 4).

Because seed transmission of BSMV could provoke biosecurity concerns, we also asked whether BSMV was transmitted to the mutated progeny (Figure 1B). Total RNA was extracted from randomly selected M1 mutants, and RT-PCR analysis revealed that 15 of 18 tested *TaGASR7* mutants were virus-free (Figure 3G and Supplemental Figure 5), indicating that BSMV tends to be eliminated by selfing, yielding progeny that are virus-free yet carry targeted mutations.

BSMV-induced chimeric mutations could be efficiently transmitted to the next generation

Since a significant portion of the M1 mutants contained chimeric mutations, we also investigated whether these mutations were transmitted to the next generation (Supplemental Figure 6 and Supplemental Table 4). We analyzed the NGS reads of some of the chimeras, and sorted the chimeric mutants into three types (Chi-1, Chi-2, and Chi-3) according to the rates of mutation at the targeted locus (Figure 4A). In Chi-1, 15.0%–35.0% of the reads per target site were mutated, in Chi-2 the proportion was 35.1%–65.0%, and in Chi-3 it was 65.1%–100.0%. Further analysis showed that about 85% of the M1 chimeric mutants in all three edited genes were Chi-2 or Chi-3, and fewer were Chi-1 (Figure 4A). Next, to see whether these chimeric mutations were transmitted to the M2 generation, we examined 48 different mutations from 18 chimeric and 4 non-chimeric M1 mutant subgenomes, including 5 Chi-1, 11 Chi-2, 9 Chi-3, 13 heterozygous, and 10 homozygous/biallelic (Supplemental Table 4). We found that very few of the Chi-1 mutations were inherited. In contrast, Chi-2 and Chi-3 showed about 70.0% and 81.0% transmission, and for heterozygous and homozygous/biallelic, it was 72.8% and 100% (Figure 4B). We also observed the highest M2 frequency of fully bleached seedlings due to loss of *PDS* function in the Bobwhite-M2-TaPDS-8-16 line, which harbored a Chi-3 mutation in the D subgenome (Figure 4C and Supplemental Tables 2 and 4). These results demonstrate that,



Targeted genes	No. of inoculated plants	No. of M0 plants with mutations	No. of M1 progenies	No.of M1 mutants/ frequency (%)	No. of M1 *abd* mutants/ frequency (%)	No. of M1 *aabbdd* mutants/ frequency (%)
TaPDS	27	24	304	180 (52.9)	97 (31.9)	5 (1.6)
TaGW2	18	14	216	148 (68.9)	46 (21.3)	0 (0)
TaGASR7	21	16	221	102 (46.2)	60 (27.1)	17 (7.7)

Figure 3. Heritable editing using BSMV-sg in three wheat varieties.

(A–C) (A) Comparison of heritable editing frequencies from the M0 to M1 generation of the BSMV-sg and BSMV-sg-mTaFT vectors at target sites of *TaPDS*, *TaGW2*, and *TaGASR7* in Bobwhite. Uninfected Bobwhite served as control. Heritable mutation frequencies of BSMV-sg and BSMV-sg-mTaFT **(B)** at *TaPDS* in Fielder and **(C)** at *TaGASR7* in Zhengmai 7698. The corresponding uninfected varieties of wheat served as controls. Heritable editing frequencies are the fraction of progenies containing a mutation in at least one subgenome of the target gene, divided by the total number of progenies by genotyped. Each dot represents the heritable editing frequency in the progeny of one parent replicate infected with BSMV-sg or BSMV-sg-mTaFT at target sites of *TaPDS*, *TaGW2*, and *TaGASR7* in three wheat varieties. The violin plot elements show the mutation frequency distribution with medians and quartiles, and significances are indicated between BSMV-sg and BSMV-sg-mTaFT by exact *P* value (ANOVA, Tukey's HSD). Data are means ± SEM.

(D) Relative proportions of the three different mutation types among the M1 mutants for *TaPDS*, *TaGW2*, and *TaGASR7*. Mutated progenies with heterozygous (Heter) mutation have one wild-type allele and one mutant allele; mutated progenies with homozygous (Ho) and biallelic (Bi) mutation have only one and two mutation alleles, respectively; mutated progenies with more than two genotypes were defined as chimeric (Chi) mutants.

(E) Numbers of infected plants and mutagenesis frequencies of the M1 progenies in three target genes. *abd* mutants showed M1 progenies with mutations in three subgenomes of corresponding target genes. *aabbdd* mutants showed M1 progenies with homozygous or biallelic mutations in three subgenomes of corresponding target genes.

(F) Phenotypes of embryo-rescued M1 lines Bobwhite-M1-TaPDS-14 and Fielder-M1-TaPDS-5 derived from parental plants infected with BSMV-sg-TaPDS. Albino shoots are indicated by red arrows. Scale bars, 2 cm.

(G) Detection of BSMV in M1 mutant progenies by RT-PCR. Total RNA was extracted from the Bobwhite-M1-TaGASR7-5 and M1-TaGASR7-10 progenies of BSMV-sg-TaGASR7-infected parental Bobwhite plants to detect BSMV using primers that amplify the BSMV genome (top) and the endogenous *TaEF1α* gene (bottom). Samples from *N. benthamiana* (Nb-TaGASR7) and wheat (M0-Bobwhite) infected by BSMV-sg-TaGASR7 were used as positive controls, and an uninfected Cas9-transgenic Bobwhite (Bob-Cas9) sample served as a negative control.

although BSMV induced a large proportion of chimeric mutations in the M1 generation, most of the edits were transmitted to the next generation.

Multiplex genome editing by BSMV-sg in wheat

Multiplex gene editing is very useful in some circumstances, for example, for deleting large DNA fragments and combining multiple mutations to improve agronomic traits (Zhu et al., 2020; Luo et al., 2021). To see if BSMV could provide multiplex gene editing, we designed a multiple gene editing strategy using a mixed *Agrobacterium* pool (MEA) (Figure 5A). Equal concentrations of *Agrobacterium* lines harboring different sgRNAs in BSMVγ-sg were mixed and co-inoculated onto *N. benthamiana* leaves with *Agrobacterium* containing BSMVα and BSMVβ, and an extract of the inoculated leaves was used to infect Cas9-transgenic wheat (Figure 5A).

We examined two- and three-gene multiplex editing with this strategy, and tested for targeted mutagenesis of endogenous

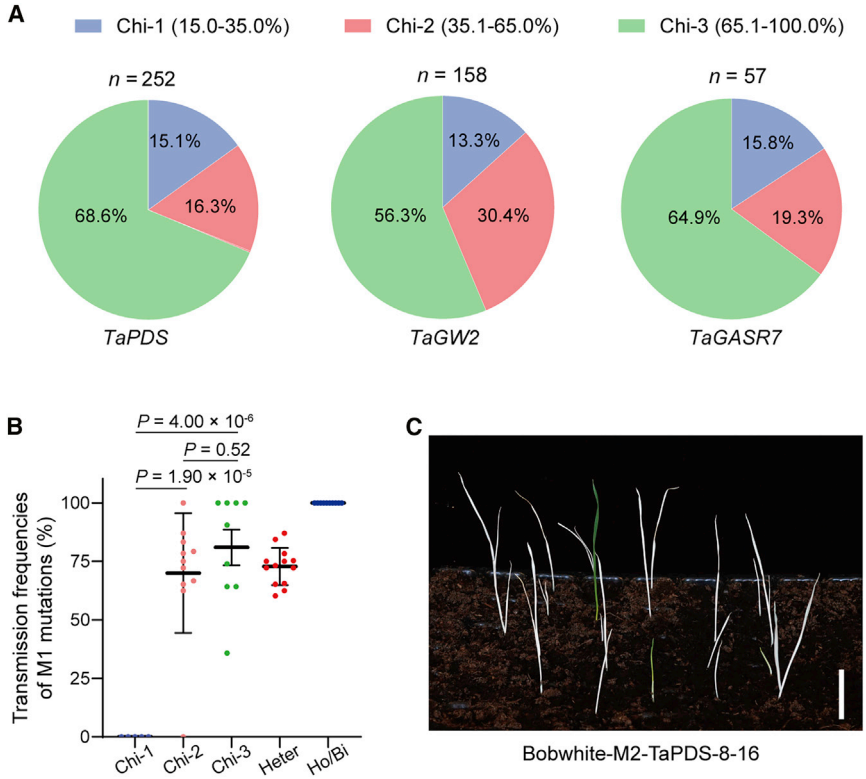


Figure 4. The transmission analysis of M1 chimeric mutations.

(A) Relative proportions of the three M1 chimeric mutation types divided by mutation ratio. *n* = 252, 158, and 57 chimeric mutations of 155, 97, and 39 M1 mutant plants from BSMV-sg-TaPDS-, BSMV-sg-TaGW2-, and BSMV-sg-TaGASR7-infected parental plants.

(B) Mutation transmission frequency of different mutation types from the M1 to the M2 generation. Chimeric mutation transmission frequencies are the fraction of M2 progenies with a mutation in the corresponding subgenome of the target gene divided by the total number of M2 progenies genotyped. Each dot represents the heritable editing frequency in M2 progeny of the subgenome of one M1 parent replicate. *n* = 5, 11, 9, 13, and 10 mutations of Chi-1, Chi-2, Chi-3, Heter (heterozygous), and Ho/Bi (homozygous/biallelic) from 22 M1 mutant plants were tested for mutation transmission analysis. Significances are indicated among different groups by exact *P* value (ANOVA, Tukey's HSD). Data are means ± SEM.

(C) Representative albino segregants among the M2 progeny derived from Bobwhite-TaPDS-8-16 with chimeric mutations in the D subgenome. Scale bar, 2 cm.

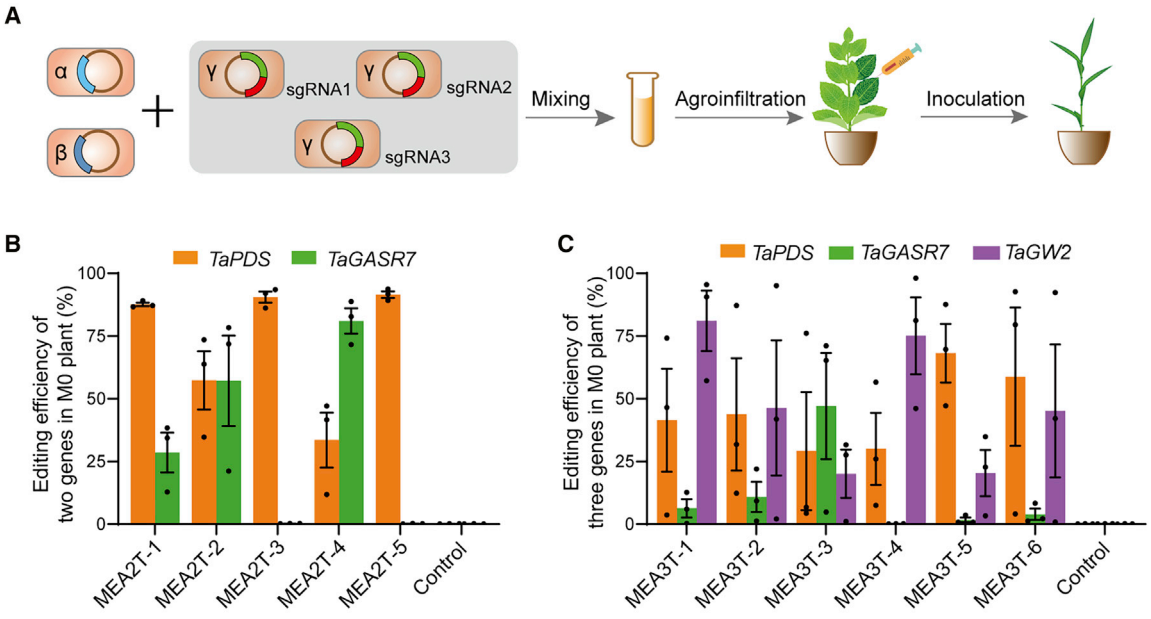
loci in symptomatic leaves infected with BSMV-sg at about 14 dpi. With *TaPDS* and *TaGASR7* as targets, three of five infected plants contained numerous mutations in *TaPDS* and *TaGASR7*. With *TaPDS*, *TaGASR7*, and *TaGW2* as targets, all three genes were multiplex edited in five of six infected plants, although mutations were less numerous at the *TaGASR7* site than in *TaPDS* and *TaGW2* (Figure 5B and 5C).

To see if the multiplex edits were heritable, we analyzed the M1 progeny of BSMV-sg-infected wheat lines of MEA2T-1, MEA2T-2, and MEA2T-4 (MEA2T group) and MEA3T-1, MEA3T-2, MEA3T-3, and MEA3T-6 (MEA3T group), which carried multiple edits in the M0 generation, as shown in Figure 5B and 5C. Simultaneous editing of *TaPDS* and *TaGASR7* was detected in the M1 progeny of all three lines of the MEA2T group, with efficiencies of 5.3%, 47.3%, and 35.7%, respectively (Figure 5D and Supplemental Figure 7 and Supplemental Table 5). For the MEA3T group, we found no progeny in which all three genes (*TaPDS*, *TaGASR7*, and *TaGW2*) had been simultaneously edited. Instead, progeny with edits in two targets (*TaPDS* and *TaGW2*) were detected, with efficiencies ranging from 11.1% to 50.0% (Figure 5D), but no targeted mutations of *TaGASR7* in the M1 progeny, possibly due in part to the low editing efficiency of *TaGASR7* in the M0 generation. Notably, one mutant with a fully bleached phenotype was homozygous for all six copies of *TaPDS* and chimeric for *TaGW2* (Figure 5E). RT-PCR analysis of randomly selected multiplex-edited M1 mutants revealed that 11 of 12 MEA2T and 5 of 9 MEA3T tested mutants were virus free (Figure 5D and Supplemental Figure 8). These results demonstrated that BSMV-sg can perform multiplex heritable editing in wheat.

Obtaining Cas9-free mutants by crossing BSMV-sg-inoculated plants with wild-type wheat

Because of the limited cargo capacity of BSMV, BSMV-sg-induced heritable gene editing depends on using Cas9-transgenic wheat, and this could lead to regulatory issues. To circumvent this problem, we devised a virus-induced transgene-free editing technique (VITF-Edit) (Figure 6A). We imagined that if BSMV could infect wheat anthers and enter the pollen, after crossing with wild-type plants, the Cas9/sgRNA complex in the pollen might edit the targeted allele not only in the sperm cells, but also in the eggs of wild-type plants. This way, we might be able to obtain mutant F1 progeny with both parental genomes edited. The Cas9 transgene could then be segregated out, thereby making it possible to obtain Cas9-free mutant progeny (Figure 6A).

To test the feasibility of VITF-Edit, we examined whether the anthers from BSMV-sg-infected Bobwhite plants had edits. Twenty to thirty anthers were pooled together as one replicate for genomic DNA extraction, and NGS showed that BSMV-sg caused many mutations, ranging from 79.6% to 97.8%, in the target sites of *TaPDS*, *TaGASR7*, and *TaGW2* in anthers (Figure 6B). Notably, the anthers from BSMV-sg-TaPDS-inoculated Bobwhite plants exhibited a photobleaching phenotype (Figure 6C). These results and RT-PCR detection of BSMV-infected wheat anthers suggest that BSMV can infect a high proportion of wheat anthers, and this can lead to editing in pollen cells (Supplemental Figure 9). We then pollinated wild-type Bobwhite plants with the pollen from BSMV-sg-TaPDS-infected Bobwhite plants, and obtained 23 F1 progeny seedlings. NGS analysis showed that targeted mutagenesis occurred in 15



D

Infected M0 plant ID	No. of M1 seedlings	No. of *TaPDS* Mutants	No. of *TaGASR7* Mutants	No. of *TaGW2* Mutants	Mutiplex editing Mutants/Ratio (%)
MEA2T-1	19	12	7	/	1 (5.3)
MEA2T-2	19	18	10	/	9 (47.3)
MEA2T-4	14	5	14	/	5 (35.7)
MEA3T-1	9	2	0	8	1 (11.1)
MEA3T-2	11	5	0	8	3 (27.3)
MEA3T-3	10	8	0	7	5 (50.0)
MEA3T-6	5	1	0	5	1 (20.0)

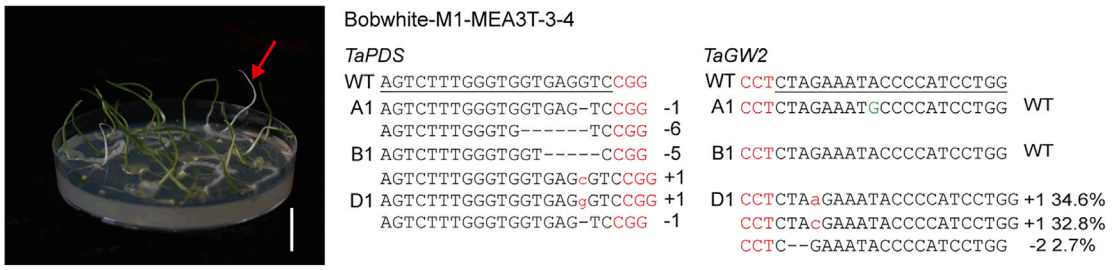


Figure 5. Multiplexed heritable gene editing using BSMV-sg in wheat.

(A) Overview of BSMV-sg-mediated multiplexed gene editing strategy using a mixed *Agrobacterium* pool (MEA). *Agrobacterium* strain derivatives harboring different BSMVγ-sg vectors were mixed at the same concentration and inoculated onto *N. benthamiana* leaves together with BSMVα and BSMVβ. Three to seven days later, inoculated *N. benthamiana* leaves were collected and homogenized with an inoculation buffer for mechanical inoculation of Cas9-transgenic wheat.

(B and C) BSMV-mediated multiplex gene editing efficiencies in M0 wheat plants. *Agrobacterium* mixtures of **(B)** BSMV-sg-TaPDS and BSMV-sg-TaGASR7 or **(C)** BSMV-sg-TaPDS, BSMV-sg-TaGASR7, and BSMV-sg-TaGW2 were inoculated onto Cas9-transgenic wheat as described for **(A)**. Uninoculated Cas9-transgenic wheat served as a control. Three different leaf samples were prepared separately from one plant, and each is shown as an individual dot.

(D) BSMV-mediated multiplexed gene editing in M1 wheat progenies. The progenies of double-gene editing lines (MEA2T-1, MEA2T-2, and MEA2T-4) and triple-gene editing lines (MEA3T-1, MEA3T-2, MEA3T-3, and MEA3T-6) were used for mutation testing.

(E) Phenotypes of M1 mutant progenies from the triple-gene-targeted wheat plants. MEA3T3-4 had a photobleaching phenotype and, correspondingly, edits were detected in all three homeoalleles of *TaPDS* (right). Scale bar, 2 cm.

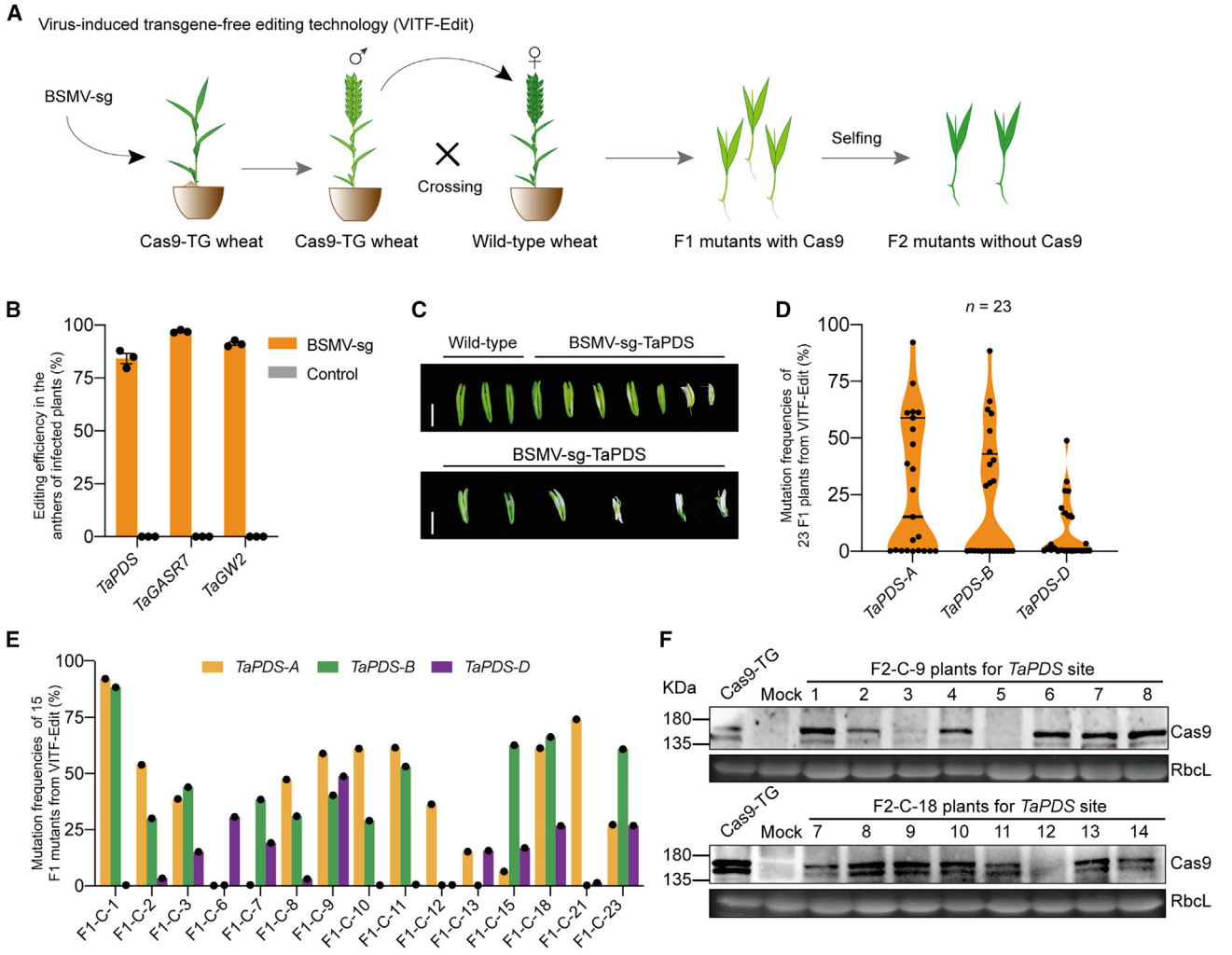


Figure 6. Obtaining Cas9-free mutants by crossing BSMV-sg-inoculated plants with wild-type wheat.

(A) Procedure of the virus-induced transgene-free editing technique (VITF-Edit). BSMV-sg was inoculated onto Cas9-transgenic (Cas9-TG) wheat plants at the jointing stage, and the infected plants were crossed with wild-type wheat after maturation, leading to F1 progeny harboring edits. After selfing of the F1 mutants, the Cas9 transgene could be segregated out.

(B) Anthers from the BSMV-sg-inoculated Cas9-transgenic wheat plants were edited with a high efficiency in three genes. Twenty to thirty anthers were collected from each inoculated plant for NGS. Anthers from uninoculated Cas9-transgenic Bobwhite plants served as controls.

(C) Photobleaching phenotypes of anthers from BSMV-sg-TaPDS-inoculated Bobwhite. Scale bars, 3 mm.

(D) Mutation frequencies at *TaPDS* in the F1 progenies from VITF-Edit. *n* = 23 F1 seedlings were used to NGS analysis.

(E) Mutation frequency of each F1 mutant from VITF-Edit.

(F) Western blot analysis of Cas9-free plants in the F2 generation. Numbers above the lanes indicate F2 seedlings used for western blot analysis. Molecular weights (in kDa) are shown on the left, and the antibody used for detection is shown on the right. Cas9-transgenic (Cas9-TG) Bobwhite samples were used as positive controls; wild-type Bobwhite samples were used as negative controls (Mock). Ribulose bisphosphate carboxylase large chain (RbcL) served as a protein loading control.

of the 23 F1 progenies, with editing frequencies ranging from 15.2% to 92.2% in *TaPDS* sites (Figure 6D). The mutagenesis frequency of seven mutants in *TaPDS-A* and five in *TaPDS-B* exceeded 50% (Figure 6D and Supplemental Figure 9 and Supplemental Table 6). In addition, 4 of the 15 F1 mutants contained targeted edits in all of the *TaPDS-A/B/D* subgenomes, and 2 mutants (F1-C-1 and F1-C-18) had targeted mutagenesis ratios exceeding 50% in both *TaPDS-A* and *TaPDS-B* (Figure 6E). These results show that Cas9/sgRNA complexes introduced into pollen cells can edit the egg cell genome.

We therefore examined whether BSMV was transmitted to the mutated progenies; total RNA was extracted from 10 randomly selected F1 mutants, and RT-PCR showed that all were virus-free (Supplemental Figure 9), indicating that BSMV could be eliminated by crossing. The F2 progeny derived from F1-C-9 and F1-C-18 self-crossing still contained targeted mutations, and transgene-free mutants were obtained after segregation by VITF-Edit (Figure 6F and Supplemental Figure 10 and Supplemental Table 7). Taken together, our results show that the VITF-Edit system can be used to generate transgene-free and virus-free genome-edited wheat plants.

DISCUSSION

To overcome the bottleneck imposed by tissue culture, methods such as *de novo* induction of meristems and RNA virus–mediated sgRNA delivery systems have been developed (Liu and Zhang, 2020; Maher et al., 2020; Oh et al., 2021). However, up to now, both of these approaches have achieved heritable gene editing only in dicotyledonous plants, and whether they can be applied to economically and agriculturally important monocot crops, such as wheat, remained to be seen. Here, we have described a BSMV-mediated genome editing system. We have shown that this system (BSMV-sg) can perform highly efficient, multiplex, and heritable gene editing in different wheat varieties, including an elite wheat variety (Zhengmai 7698), without the need for transformation and tissue-culture procedures. A Cas9-transgenic wheat line has to be created first, and the Cas9-transgene and virus can be subsequently eliminated by backcrossing to the wild type. We thus describe, for the first time, highly efficient heritable genome editing in monocotyledonous plants using a viral vector. After the successful addition of mobile RNA elements to a TRV-based vector to create heritable gene edits (Ali et al., 2015; Ellison et al., 2020), other viruses such as PVX and a CLCrV were also used to achieve heritable gene editing in model dicots by a similar strategy (Lei et al., 2021; Uranga et al., 2021). However, introduction of either *FT* RNA or tRNA, or both, into the BSMV-sg system failed to improve the efficiency of heritable gene editing. This result implied that the effects of *FT* RNA and tRNA on heritable gene editing might be somewhat dependent on the viral vector used. In fact, BSMV is able to enter the apical meristems of root and shoot tips of systemically infected wheat (Lin and Langenberg, 1984), which is distinct from other viruses. There is evidence that meristem entry of virus is directly correlated to its subsequent infection of reproductive organs and seeds (Bradamante et al., 2021). Indeed, BSMV is a typical seed-borne virus, which is able to infect the pollens, ovules, and embryos of barley (Carroll, 1972; Carroll and Mayhew, 1976). Recently, Raz et al. reported that BSMV-based virus-induced gene silencing could be used to study genes involved in meiotic recombination in wheat, which is another proof supporting germline transmission events during BSMV infection (Raz et al., 2021). Altogether, these unique properties enable BSMV to deliver the inserted sgRNAs into the meristem cells and generate heritable gene editing in the Cas9-transgenic wheat plants.

We often observed that seed transmissibility of BSMV is positively associated with its ability to achieve heritable gene editing. The RNAγ plays a major role in the seed transmissibility of BSMV (Edwards, 1995). As the inserted sgRNA and mobile RNA elements are immediately adjacent to the tRNA-like structure, an important element within the RNAγ that functions in the replication, movement, and seed transmissibility of BSMV (Kozlov Yu et al., 1984; Zhou and Jackson, 1996; Edwards, 1995), we speculate that the tRNA and/or FT-RNA elements may interfere with the BSMV tRNA-like structure, thus impairing the infection or seed transmissibility of BSMV and leading to a decreased frequency of heritable gene editing. In addition, we also tested these mobile RNA elements in Cas9-transgenic *N. benthamiana* using BSMV-sg-mAtFT, BSMV-sg-mTaFT, and BSMV-sg-tRNA, but none of them generated heritable gene editing in the progenies (Supplemental Figure 11). These results suggested that the introduction of these mobile RNA elements into the BSMV-based vector also did not help to produce the heritable gene editing in *N. benthamiana*.

Heritable gene editing rates ranging from 12.9% to 100.0% can be achieved using the BSMV-sg system—much higher than by using the conventional methods reported so far in wheat (Zhang et al., 2016b; Gao, 2021). The growth stage of wheat for BSMV inoculation was changed to the jointing stage, compared with the two-leaf stage before (Hu et al., 2019), which greatly alleviates the detrimental effect of BSMV infection on wheat growth and sets more seeds for mutant screening. In particular, it should be pointed out that targeted modifications in all three homeoalleles can frequently be detected in the M1 progenies. Another interesting phenomenon is that a significant portion of the M1 progenies contained chimeric mutations, and the edits were also heritable in more than 80% of such cases, suggesting that most of the chimeras may be generated by BSMV-sg-mediated editing of developing embryos rather than somatic cells. In addition, the total time required for obtaining mutated M1 wheat progenies based on the BSMV-sg system was about 6–8 weeks from inoculation of the M0 wheat plants to obtaining the M1 mutants by embryo rescue. Therefore, our system can significantly reduce genome editing time in wheat compared with the usual 3 months minimum.

Multiplexed gene editing is of great importance in wheat molecular breeding for pyramiding multiple traits (Zhu et al., 2020). In this study, we achieved multiplex and heritable gene editing simply by co-inoculation of multiple BSMVγ-sg vectors onto wheat leaves. This is different from TRV-based multiplex gene editing, which requires the simultaneous expression of different sgRNAs in an individual TRV vector to avoid superinfection exclusion (Ellison et al., 2020). To test whether superinfection exclusion occurs in BSMV, *N. benthamiana* leaves were inoculated with *Agrobacterium* mixtures of BSMVα, BSMVβ, BSMVγ-γb-GFP, and BSMVγ-γb-mCherry. Inoculated leaves were sampled for confocal microscopy analysis at 3 dpi. GFP and mCherry fluorescence can be observed in a cell simultaneously, indicating that different BSMV derivatives can infect the same cells (Supplemental Figure 12). Our system makes combining different sgRNAs for implementing multiplexed gene editing more flexible and convenient.

Since the cargo of editing with BSMV-sg is limited, editing depends on the use of Cas9-transgenic wheat, but we were able to eliminate the transgene via the VITF-Edit method established in this work. Interestingly, NGS analysis showed that some of the F1 mutants were chimeras, with gene editing efficiencies of exceeding 50%, implying that the Cas9/sgRNA complexes may be retained in developing embryos after fertilization.

In conclusion, we have developed a BSMV-based system that greatly simplifies CRISPR-Cas9-based editing in wheat. BSMV infects many other agronomically important cereal crops, such as barley (*Hordeum vulgare*), maize (*Zea mays*), millet (*Setaria italica*), and oats (*Avena sativa*) (Jackson et al., 2009; Yuan et al., 2011; Hu et al., 2019). This system provides a basis for expanding the utility of the BSMV-sg described here to other crop species. Furthermore, we envision that this approach should be applicable to creating precise gene edits by infecting

transgenic wheat plants with other types of gene editing constructs such as base editors (Zhu et al., 2020). Because of its efficiency, ease of use, and low cost, we believe that the BSMV-sg system will provide an attractive tool for carrying out large-scale and high-throughput genome editing in wheat.

METHODS

Plant materials and growth conditions

N. benthamiana plants were grown in a greenhouse under 15/9 h light/dark conditions at 23°C–25°C, as previously described (Yuan et al., 2011). Wheat seedlings were grown in a greenhouse under 16/8 h light/dark conditions at 20°C–23°C, and inoculated plants were maintained in the same climate-controlled chamber for subsequent seed setting and embryo rescue. Seedlings of Zhengmai 7698 at the two-leaf stage were kept at 4°C for 2 weeks for vernalization.

Vector construction

To construct the BSMVγ-sg vector containing different sgRNAs, an intermediate vector, pCB301-BSMVγ-SmR, was generated, as described previously (Hu et al., 2019). To add the tRNA of methionine, mTaFT, or mAtFT to the 3′ end of the sgRNA, several intermediate vectors were constructed prior to insertion of the specific sgRNA. Briefly, pCB301-BSMVγ-SmR was linearized by inverse PCR downstream of the second *Sap*I site, and then tRNA, mTaFT, or mAtFT fragments were amplified with cDNA templates prepared from wheat or *Arabidopsis* and assembled into linearized pCB301-BSMVγ-SmR to create the intermediate vectors pCB301-BSMVγ-sg-tRNA and pCB301-BSMVγ-sg-mTaFT/mAtFT. Oligo pairs (forward, 5′-CTAN$_{(20)}$-3′; reverse, 5′-AACN$_{(20)}$-3′) were annealed and ligated into the aforementioned four pCB301-BSMVγ-sgRNA vector backbones that had been digested with *Sap*I. The target site of *TaPDS* was inserted into these four intermediate vectors, yielding BSMVγ-TaPDS, BSMVγ-TaPDS-tRNA, BSMVγ-TaPDS-mAtFT, and BSMVγ-TaPDS-mTaFT. The target sites of *TaGW2* and *TaGASR7* were introduced into BSMVγ-sg and BSMVγ-mTaFT, resulting in BSMVγ-TaGW2, BSMVγ-TaGW2-mTaFT, BSMVγ-TaGASR7, and BSMVγ-TaGASR7-mTaFT.

To insert tRNA-sgRNA into pCB301-BSMVγ-sg, pCB301-BSMVγ-tRNA, and pCB301-BSMVγ-mTaFT, tRNA-target-sgRNA was amplified using a tRNA-sgRNA scaffold intermediate vector by designing three specific primers. The target site of *TaPDS* was built into these three vectors, yielding BSMVγ-tRNA-TaPDS, BSMVγ-tRNA-TaPDS-mTaFT, and BSMVγ-tRNA-TaPDS-tRNA. All of the sgRNA target sites, oligonucleotides, and mobile RNA sequences used in this study are listed in Supplemental Table 8.

BSMV infection of wheat

BSMV-derived plasmids were transformed into *Agrobacterium tumefaciens* strain EHA105 for agroinfiltration of *N. benthamiana* leaves. Equal volumes of *Agrobacterium* lines harboring the BSMVα, BSMVβ, and BSMVγ-sg derivatives expressing the sgRNA were mixed to a final A_{600} of 0.3 for each construct, and co-infiltrated into leaves of 4- to 6-week-old *N. benthamiana* plants, as described previously (Yuan et al., 2011); 3–7 dpi, the inoculated leaves were collected and homogenized in a mortar with an inoculation buffer containing 10 mM sodium phosphate and 0.5% freshly prepared sodium sulfite in a ratio of 1:5 (w/v). In this study, wheat plants grown to jointing stage were used for rub-inoculation, and two newly expanded leaves were mechanically inoculated with the tissue homogenates.

For multiplex gene editing of dual-target groups, equal (A_{600} 0.3) concentrations of the different BSMVγ-sg-containing *Agrobacterium* were mixed, while for the triple-target groups, A_{600} was 0.2. The *Agrobacterium* was infiltrated into the same *N. benthamiana* leaves together with *Agrobacterium* harboring BSMVα and BSMVβ at A_{600} 0.6. At 3–7 dpi, infiltrated leaves were harvested and homogenized for wheat inoculation.

Wheat hybridization

Cas9-transgenic Bobwhite and wild-type Bobwhite seeds were sown at the same time. Cas9-transgenic Bobwhite plants were inoculated with BSMV-sg-TaPDS at the jointing stage, when the spike of wild-type Bobwhite was just fully emerged from the flag leaf and would thus be ready for emasculation. Then the central florets and remaining floret anthers of each spikelet were removed by scissors and forceps. The emasculated spike was covered with a crossing bag to avoid any cross-pollination and secured with a stapler. Three to seven days after emasculation of wild-type Bobwhite, the anthers of BSMV-infected Cas9-transgenic Bobwhite were collected for artificial pollination. After 3–4 weeks, the immature embryos of crossing plants could be rescued for further genotyping analysis.

RT-PCR analysis of BSMV in wheat progenies

For RT-PCR detection of BSMV in wheat progenies, total RNA was extracted using Trizol reagent (Thermo Fisher Scientific) and quantified with a NanoDropND-1000 (Thermo Fisher Scientific); cDNA was prepared from 2 μg of DNase-treated total RNA using an oligo-dT primer and a BSMV 3′-UTR-specific primer (BS32), and a fragment corresponding to the BSMV 3′ UTR was amplified using the primer pair BS32/BSMV-3U-F. A *TaEF1α*-specific fragment was amplified using the primer pair TaEF1α-F/R (Zhang et al., 2013) and served as a control.

Plant sample collection and DNA extraction

To capture all possible induced mutations due to the ongoing effects of BSMV-sg, three different leaf samples were collected from each of the BSMV derivative-infected plants and M1 progeny and mixed, and the genomic DNA of the corresponding progenies was extracted by the cetyltrimethylammonium bromide method (Doyle and Doyle, 1990). For the editing efficiency evaluation of the mixed BSMV-sg-infected plants, three different leaf samples were prepared separately from one plant as replicates. For the editing efficiency evaluation of BSMV-sg-infected wheat pollen, 20–30 anthers were collected from each inoculated plant as replicates. Genomic DNA should be extracted from symptomatic systemic wheat leaves at least 15 days after viral inoculation.

Analysis of mutation frequencies and genotyping

A conserved primer set that recognizes all three homeoalleles of *TaPDS*, *TaGASR7*, and *TaGW2*, respectively, was used to amplify the corresponding sgRNA target sites of infected M0 plants to evaluate the mutation rates induced by different BSMV vectors. PCR restriction-enzyme digestion assays were performed to preliminarily identify mutations in the target genes of M1 and M2 plants, as described previously (Shan et al., 2014). For further identification of the types of mutations in the mutant M1 plants, deep amplicon sequencing was performed after two rounds of PCR. In the first round, the target regions were amplified using the M1 genomic DNA as template and primers specific for subgenome A, B, or D. In the second round, forward and reverse barcodes were added to the ends of the PCR products using designed primers, with the first-round PCR products as templates. Equal amounts of the second-round PCR products were mixed as a pool, purified using a gel purification kit, and sequenced commercially with the Illumina NovaSeq 6000 platform (Novogene). Paired-end .fastq files from NGS were analyzed as previously described (Li et al., 2020). The sgRNA target sites in the sequenced reads were examined to identify mutation types and indel (insertion and deletion) ratios. Due to the noise in NGS-based indel detection and the continuous movement and expression of BSMV-derived sgRNAs, we used the following definitions to designate the genotypes of each subgenome: 0%–15.0% indels, WT; 35.1%–65.0% indels with only one mutation type, heterozygotes; 85.1%–100.0% indels, homozygotes with one mutation type and biallelic with two; and 15.1%–35.0%, 65.1%–85.0%, and more than two genotypes were regarded as

chimeric mutations. The second-round primers with different barcodes of NGS are listed in Supplemental Table 9.

Rescue of wheat immature embryos

Whole spikes with stalks of BSMV-infected or crossed wheat were excised at 15–30 days after pollination, sterilized in 75% ethanol for 1 min, and washed three times with sterile water. Green wheat seeds were threshed from the spikes and further sterilized with 4% NaClO for 25–30 min. After being washed three times with sterile water, the immature embryos were carefully stripped from the green seeds using forceps and plated onto wheat rooting medium in 90-mm-diameter Petri dishes with the scutellum side face up. Isolated embryos were incubated in the dark at 23°C for 3 days to sprout, followed by cultivation under a 16/8 h light/dark regime for 5 days. The leaves were then collected for genotype analysis.

Statistical analysis

For quantitative analysis of the viral infection rate, somatic editing frequency, mutation frequency from M0 to M1 generation, and chimeric M1 mutation transmission frequency, at least three biological replicates or individual plants for each experiment were used for statistical analysis. The statistical analysis was performed with Tukey's *post hoc* ANOVA for multiple comparison. Statistical comparisons were made with the commercially available software SPSS (IBM SPSS Statistics 22). All numerical values are presented as means ± SEM.

Data availability

All data supporting the findings of this study are available in the article or in supplemental files or are available from the corresponding author upon request. The NCBI GenBank identifiers are KJ697755 (*TaGW2*), FJ517553 (*TaPDS*), and KJ000052 (*TaGASR7*). High-throughput sequencing data have been deposited in an NCBI BioProject database (accession code PRJNA729216).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

Y.W. and Y.Z. conceived the project and designed the experiments. T.L., J.H., Y.S., B.L., D.Z., W.L., and J.L. performed the experiments. Y.W., Y.Z., T.L., and J.H. analyzed the data and interpreted the results. C.G. and D.L. supervised the research and provided suggestions. Y.W., Y.Z., T.L., and J.H. wrote the manuscript.

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REFERENCES

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

Ali, Z., Abul-faraj, A., Li, L., Ghosh, N., Piatek, M., Mahjoub, A., Aouida, M., Piatek, A., Baltes, N.J., Voytas, D.F., et al. (2015). Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol. Plant **8**:1288–1291.

Altpeter, F., Springer, N.M., Bartley, L.E., Blechl, A.E., Brutnell, T.P., Citovsky, V., Conrad, L.J., Gelvin, S.B., Jackson, D.P., Kausch, A.P., et al. (2016). Advancing crop transformation in the era of genome editing. Plant Cell **28**:1510–1520.

Annamalai, P., and Rao, A.L. (2005). Replication-independent expression of genome components and capsid protein of brome mosaic virus in planta: a functional role for viral replicase in RNA packaging. Virology **338**:96–111.

Atkins, P.A., and Voytas, D.F. (2020). Overcoming bottlenecks in plant gene editing. Curr. Opin. Plant Biol. **54**:79–84.

Bennypaul, H.S., Mutti, J.S., Rustgi, S., Kumar, N., Okubara, P.A., and Gill, K.S. (2012). Virus-induced gene silencing (VIGS) of genes expressed in root, leaf, and meiotic tissues of wheat. Funct. Integr. Genomics **12**:143–156.

Bradamante, G., Mittelsten Scheid, O., and Incarbone, M. (2021). Under siege: virus control in plant meristems and progeny. Plant Cell, koab140. https://doi.org/10.1093/plcell/koab140.

Carroll, T.W. (1972). Seed transmissibility of two strains of *Barley stripe mosaic virus*. Virology **48**:323–336.

Carroll, T.W., and Mayhew, D.E. (1976). Occurrence of virions in developing ovules and embryo sacs of barley in relation to the seed transmissibility of *Barley stripe mosaic virus*. Can. J. Bot. **54**:2497–2512.

Chen, K., Wang, Y., Zhang, R., Zhang, H., and Gao, C. (2019). CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. **70**:667–697.

Cody, W.B., and Scholthof, H.B. (2019). Plant virus vectors 3.0: transitioning into synthetic genomics. Annu. Rev. Phytopathol. **57**:211–230.

Cody, W.B., Scholthof, H.B., and Mirkov, T.E. (2017). Multiplexed gene editing and protein overexpression using a *Tobacco mosaic virus* viral vector. Plant Physiol. **175**:23–35.

Debernardi, J.M., Tricoli, D.M., Ercoli, M.F., Hayta, S., Ronald, P., Palatnik, J.F., and Dubcovsky, J. (2020). A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat. Biotechnol. **38**:1274–1279.

Doyle, J.J., and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. Focus **12**:13–15.

Edwards, M.C. (1995). Mapping of the seed transmission determinants of *Barley stripe mosaic virus*. Mol. Plant Microbe Interact. **8**:906–915.

Ellison, E.E., Nagalakshmi, U., Gamo, M.E., Huang, P.J., Dinesh-Kumar, S., and Voytas, D.F. (2020). Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. Nat. Plants **6**:620–624.

Gao, C. (2021). Genome engineering for crop improvement and future agriculture. Cell **184**:1621–1635.

Gao, Q., Xu, W.Y., Yan, T., Fang, X.D., Cao, Q., Zhang, Z.J., Ding, Z.H., Wang, Y., and Wang, X.B. (2019). Rescue of a plant cytorhabdovirus as versatile expression platforms for planthopper and cereal genomic studies. New Phytol. **223**:2120–2133.

Holzberg, S., Brosio, P., Gross, C., and Pogue, G.P. (2002). *Barley stripe mosaic virus*-induced gene silencing in a monocot plant. Plant J. **30**:315–327.

Hu, J., Li, S., Li, Z., Li, H., Song, W., Zhao, H., Lai, J., Xia, L., Li, D., and Zhang, Y. (2019). A *Barley stripe mosaic virus*-based guide RNA delivery system for targeted mutagenesis in wheat and maize. Mol. Plant Pathol. **20**:1463–1474.

Jackson, A.O., Lim, H.S., Bragg, J., Ganesan, U., and Lee, M.Y. (2009). Hordeivirus replication, movement, and pathogenesis. Annu. Rev. Phytopathol. **47**:385–422.

Jiang, N., Zhang, C., Liu, J.Y., Guo, Z.H., Zhang, Z.Y., Han, C.G., and Wang, Y. (2019). Development of *Beet necrotic yellow vein virus*-based vectors for multiple-gene expression and guide RNA delivery in plant genome editing. Plant Biotechnol. J. **17**:1302–1315.

Kaya, H., Ishibashi, K., and Toki, S. (2017). A split *Staphylococcus aureus* Cas9 as a compact genome-editing tool in plants. Plant Cell Physiol. **58**:643–649.

Kong, J., Martin-Ortigosa, S., Finer, J., Orchard, N., Gunadi, A., Batts, L.A., Thakare, D., Rush, B., Schmitz, O., Stuiver, M., et al. (2020). Overexpression of the transcription factor GROWTH-REGULATING FACTOR5 improves transformation of dicot and monocot species. Front. Plant Sci. **11**:572319.

Kozlov Yu, V., Rupasov, V.V., Adyshev, D.M., Belgelarskaya, S.N., Agranovsky, A.A., Mankin, A.S., Morozov, S., Dolja, V.V., and Atabekov, J.G. (1984). Nucleotide sequence of the 3'-terminal tRNA-like structure in *Barley stripe mosaic virus* genome. Nucleic Acids Res. **12**:4001–4009.

Lei, J., Dai, P., Li, Y., Zhang, W., Zhou, G., Liu, C., and Liu, X. (2021). Heritable gene editing using *FT* mobile guide RNAs and DNA viruses. Plant Methods **17**:20.

Li, C., Zhang, K., Zeng, X., Jackson, S., Zhou, Y., and Hong, Y. (2009). A *cis* element within *Flowering locus T* mRNA determines its mobility and facilitates trafficking of heterologous viral RNA. J. Virol. **83**:3540–3548.

Li, C., Zhang, R., Meng, X., Chen, S., Zong, Y., Lu, C., Qiu, J.L., Chen, Y.H., Li, J., and Gao, C. (2020). Targeted, random mutagenesis of plant genes with dual cytosine and adenine base. Nat. Biotechnol. **38**:875–882.

Lin, N.S., and Langenberg, W.G. (1984). Distribution of *Barley stripe mosaic virus* protein in infected wheat root and shoot tips. J. Gen. Virol. **65**:2217–2224.

Liu, H., and Zhang, B. (2020). Virus-based CRISPR/Cas9 genome editing in plants. Trends Genet. **36**:810–813.

Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Cho, M.J., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., et al. (2016). Morphogenic regulators *Baby boom* and *Wuschel* improve monocot transformation. Plant Cell **28**:1998–2015.

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

Ma, M., Yan, Y., Huang, L., Chen, M., and Zhao, H. (2012). Virus-induced gene-silencing in wheat spikes and grains and its application in functional analysis of HMW-GS-encoding genes. BMC Plant Biol. **12**:141.

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

Maher, M.F., Nasti, R.A., Vollbrecht, M., Starker, C.G., Clark, M.D., and Voytas, D.F. (2020). Plant gene editing through *de novo* induction of meristems. Nat. Biotechnol. **38**:84–89.

Mei, Y., Beernink, B.M., Ellison, E.E., Konecna, E., Neelakandan, A.K., Voytas, D.F., and Whitham, S.A. (2019). Protein expression and gene editing in monocots using *Foxtail mosaic virus* vectors. Plant Direct **3**:e00181.

Oh, Y., Kim, H., and Kim, S.G. (2021). Virus-induced plant genome editing. Curr. Opin. Plant Biol. **60**:101992.

Peng, X., Ma, X., Lu, S., and Li, Z. (2020). A versatile plant rhabdovirus-based vector for gene silencing, miRNA expression and depletion, and antibody production. Front. Plant Sci. **11**:627880.

Ran, Y., Liang, Z., and Gao, C. (2017). Current and future editing reagent delivery systems for plant genome editing. Sci. China Life Sci. **60**:490–505.

Raz, A., Dahan-Meir, T., Melamed-Bessudo, C., Leshkowitz, D., and Levy, A.A. (2021). Redistribution of meiotic crossovers along wheat chromosomes by virus-induced gene silencing. Front. Plant Sci. **11**:635139.

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

Uranga, M., Aragones, V., Selma, S., Vazquez-Vilar, M., Orzaez, D., and Daros, J.A. (2021). Efficient Cas9 multiplex editing using unspaced sgRNA arrays engineering in a *Potato virus X* vector. Plant J. **106**:555–565.

Wang, M., Gao, S., Zeng, W., Yang, Y., Ma, J., and Wang, Y. (2020). Plant virology delivers diverse toolsets for biotechnology. Viruses **12**:1338.

Wang, R., Yang, X., Wang, N., Liu, X., Nelson, R.S., Li, W., Fan, Z., and Zhou, T. (2016). An efficient virus-induced gene silencing vector for maize functional genomics research. Plant J. **86**:102–115.

Yuan, C., Li, C., Yan, L., Jackson, A.O., Liu, Z., Han, C., Yu, J., and Li, D. (2011). A high throughput *Barley stripe mosaic virus* vector for virus induced gene silencing in monocots and dicots. PLoS One **6**:e26468.

Zhang, K., Niu, S., Di, D., Shi, L., Liu, D., Cao, X., Miao, H., Wang, X., Han, C., Yu, J., et al. (2013). Selection of reference genes for gene expression studies in virus-infected monocots using quantitative real-time PCR. J. Biotechnol. **168**:7–14.

Zhang, W., Thieme, C.J., Kollwig, G., Apelt, F., Yang, L., Winter, N., Andresen, N., Walther, D., and Kragler, F. (2016a). tRNA-related sequences trigger systemic mRNA transport in plants. Plant Cell **28**:1237–1249.

Zhang, X., Kang, L., Zhang, Q., Meng, Q., Pan, Y., Yu, Z., Shi, N., Jackson, S., Zhang, X., Wang, H., et al. (2020). An RNAi suppressor activates in planta virus-mediated gene editing. Funct. Integr. Genomics **20**:471–477.

Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Qiu, J.L., and Gao, C. (2016b). Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. **7**:12617.

Zhao, F., Lim, S., Igori, D., Yoo, R.H., Kwon, S.Y., and Moon, J.S. (2016). Development of tobacco ringspot virus-based vectors for foreign gene expression and virus-induced gene silencing in a variety of plants. Virology **492**:166–178.

Zhou, H., and Jackson, A.O. (1996). Analysis of *cis*-acting elements required for replication of *Barley stripe mosaic virus* RNAs. Virology **219**:150–160.

Zhu, H., Li, C., and Gao, C. (2020). Applications of CRISPR-Cas in agriculture and plant biotechnology. Nat. Rev. Mol. Cell Biol. **21**:661–677.