nature biotechnology

Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew

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Sequence-specific nucleases have been applied to engineer targeted modifications in polyploid genomes[1], but simultaneous modification of multiple homoeoalleles has not been reported. Here we use transcription activator–like effector nuclease (TALEN)[2,3] and clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 (refs. 4,5) technologies in hexaploid bread wheat to introduce targeted mutations in the three homoeoalleles that encode MILDEW-RESISTANCE LOCUS (MLO) proteins[6]. Genetic redundancy has prevented evaluation of whether mutation of all three *MLO* alleles in bread wheat might confer resistance to powdery mildew, a trait not found in natural populations[7]. We show that TALEN-induced mutation of all three *TaMLO* homoeologs in the same plant confers heritable broad-spectrum resistance to powdery mildew. We further use CRISPR-Cas9 technology to generate transgenic wheat plants that carry mutations in the *TaMLO-A1* allele. We also demonstrate the feasibility of engineering targeted DNA insertion in bread wheat through nonhomologous end joining of the double-strand breaks caused by TALENs. Our findings provide a methodological framework to improve polyploid crops.

Bread wheat (*Triticum aestivum* L., 2*n* = 42, AABBDD) is a major staple crop worldwide. It provides approximately 20% of all calories consumed by humans (http://faostat.fao.org/site/339/default.aspx). Given the economic importance of wheat, new traits have always been sought to improve its yield and quality, as well as its capacity to adapt to biotic and abiotic stresses. Most of these efforts have involved classical breeding. Bread wheat is an allohexaploid, with three similar but not identical copies of most of its genes[8]. Its large genome (17,000 megabases), high ploidy and high (80–90%) content of repetitive DNA make it unusually recalcitrant to forward and reverse genetic analyses[9]. Therefore, technologies that can target one specific copy or several homoeologous gene copies are needed to study and improve the agronomic traits of bread wheat.

Sequence-specific nucleases (SSNs), including zinc-finger nucleases (ZFNs)[10], TALENs, and the RNA-guided nuclease Cas9 (CRISPR-Cas9 system), have been developed to generate targeted DNA double-strand breaks, which are then repaired mainly by either error-prone nonhomologous end joining (NHEJ) or high-fidelity homologous recombination[11]. All three types of SSN have been used to create targeted gene knockouts in various plant species[12]. Although heritable gene modification has been demonstrated in *Arabidopsis*[13–15] and rice[16] using the SSNs, germline transmission of a mutation created by such genome editing strategies has not yet been achieved in hexaploid bread wheat. We have previously demonstrated genome editing in bread wheat using a transient protoplast expression system[17]. Here we report the use of TALENs and the CRISPR-Cas9 system to modify an endogenous wheat gene and produce newly introduced, stably transmitted genetic traits. Our findings underscore the potential of SSNs as efficient tools for wheat research and breeding.

We chose to target three *MLO* loci, which encode proteins that were shown to repress defenses against powdery mildew diseases in other plants[18]. Loss-of-function *mlo* alleles in barley[19], *Arabidopsis*[20] and tomato[21] lead to broad-spectrum and durable resistance to the fungal pathogens that cause powdery mildew in these species. In wheat, powdery mildew is caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), which is one of the most destructive plant pathogens worldwide. Modification of *MLO* genes in wheat may provide the opportunity to breed varieties with broad-spectrum and durable resistance to *Bgt*. The three *MLO* homoeologs in bread wheat (*TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1*) are 98% and 99% identical at the nucleotide and protein levels, respectively. The demonstration that *TaMLO-B1* can restore susceptibility to powdery mildew in a barley *mlo* mutant indicates that the function of these *MLO* genes has been conserved during evolution[6]. However, to date, no spontaneous or induced *mlo* mutants have been reported in bread wheat, probably owing to its hexaploid nature and the inherent difficulty in mutating all three *MLO* homoeoalleles in the same plant.

To modify all three *TaMLO* copies, we used a pair of TALENs (T-MLO) that target a conserved region in exon 2 (**Fig. 1a**). The TALEN pair recognizes 16-bp and 17-bp sequences of contiguous DNA separated by an 18-bp spacer DNA that contains an AvaII restriction site (**Fig. 1a** and **Supplementary Table 1**). The TALEN recognition sequences are strictly conserved in *TaMLO-B1* and *TaMLO-D1*, but have one nucleotide mismatch with the cognate

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Received 2 April; accepted 27 June; published online 20 July 2014; doi:10.1038/nbt.2969

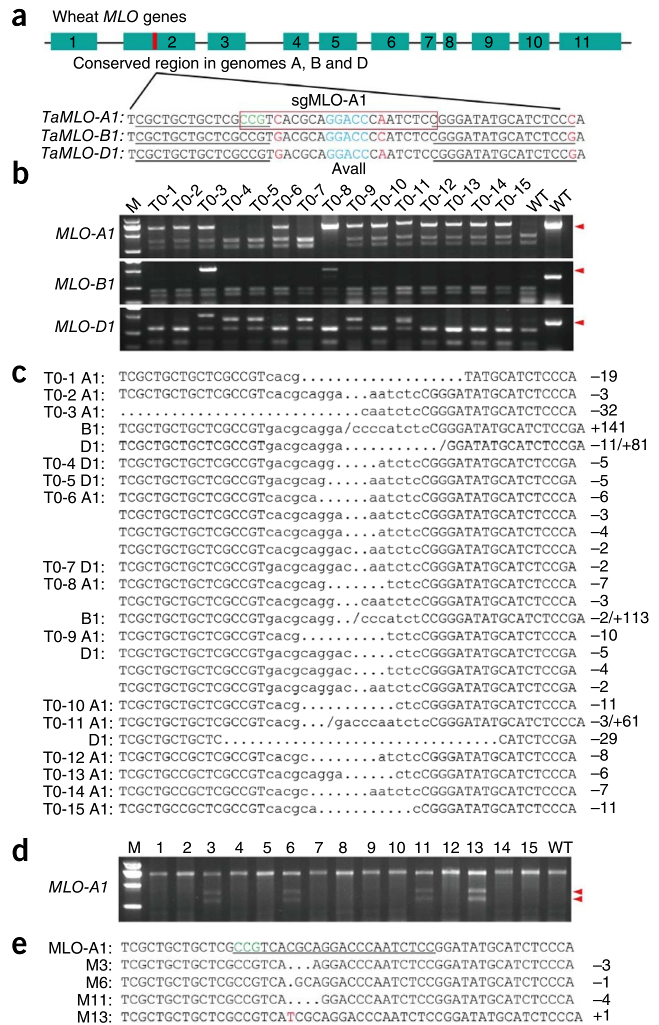

Figure 1 Targeted knockout of *TaMLO* genes using the TALEN and CRISPR-Cas9 systems. (**a**) Sites within a conserved region of exon 2 of wheat *TaMLO* homoeologs targeted by the TALEN and CRISPR-Cas9 systems. The TALEN-targeted sequences in *MLO-A1*, *MLO-B1* and *MLO-D1* are underlined, and the AvaII restriction site in the spacer is blue. Of the three SNPs highlighted in red, two are in the spacer region and one lies near the far right of the TALEN binding site. The CRISPR-Cas9 targeted sequence in *MLO-A1* is indicated in the box, and the protospacer-adjacent motif (PAM) sequence is highlighted in green. (**b**) Outcome of PCR-RE assay to detect TALEN-induced mutations in 15 representative T0 transgenic wheat plants. Mutations were identified in *TaMLO* genes amplified with gene-specific primers from independent seedlings. Lanes T0-1 to T0-15 show PCR fragments amplified from the transgenic wheat plants digested with AvaII. Lanes labeled WT show PCR fragments amplified from a wild-type control plant with or without AvaII digestion. The bands marked by red arrowheads are caused by TALEN-induced mutations. (**c**) TALEN-induced mutant *TaMLO* alleles identified by sequencing 15 representative transgenic wheat plants. The numbers on the right show the type of mutation and how many nucleotides are involved, with "–" and "+" indicating deletion or insertion of the given number of nucleotides, respectively. (**d**) Outcome of T7E1 assay to detect CRISPR-induced mutations in 15 representative T0 transgenic wheat plants. Red arrowheads indicate the fragments digested by T7E1. (**e**) Mutations in the *TaMLO-A1* site that were induced by sgMLO-A1.

TaMLO-A1 target site (**Fig. 1a**). In addition, the spacer region contains two single-nucleotide polymorphisms (SNPs) among the three *MLO* homoeoalleles (**Fig. 1a**). The TALENs were assembled by the Golden Gate cloning method[22], and built into a single plasmid by a T2A translational skipping sequence driven by the maize *Ubiquitin 1* promoter (**Supplementary Fig. 1a**). The activity of the resulting T-MLO was first evaluated by transforming the TALEN-carrying plasmid into wheat protoplasts. Analysis of genomic DNA from the transformed protoplasts using a PCR restriction enzyme digestion assay (PCR-RE)[23] demonstrated the occurrence of insertion and/or deletion (indel) mutations at the target site with efficiencies ranging from 23% to 38% in genomes A, B and D (**Supplementary Fig. 1b**,**c**). The one-nucleotide mismatch did not affect T-MLO cleavage at the *TaMLO-A1* site, probably because of the degeneracy of NN repeats, which can recognize C, A and G[2,3].

Next we co-transformed the T-MLO plasmid and pAHC20 (ref. 24), a plasmid harboring the selectable *bar* gene, into immature wheat embryos by the particle bombardment method. Wheat seedlings were regenerated from herbicide-resistant calli after 6–8 weeks of selection on 5 µg/ml phosphinothricin (PPT). The *MLO* target sites (in *TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1*) were first amplified from the genomic DNA of these transgenic seedlings (T0 plants) using a conserved primer set (**Supplementary Table 2**), and analyzed by the PCR-RE assay to detect potential mutations. We identified 27 mutations in 450 independent T0 transgenic lines (6.0%) from five independent transformation experiments in winter wheat variety Kenong199, and 8 mutations out of 237 T0 lines (3.4%) in spring wheat variety Bobwhite from one transformation experiment (**Supplementary Table 3**). In order to identify the *TaMLO* genes in which the mutations occurred, we designed primers to specifically amplify *TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1,* respectively. PCR-RE assays with the specific primers (**Supplementary Table 2**) revealed that T-MLO–induced mutations occurred in all three diploid genomes (**Fig. 1b** and **Supplementary Fig. 2**). Confirmation of the mutations by sequencing showed that most of the mutations within the TALEN target region were small deletions of 1–10 bp (**Fig. 1c** and **Supplementary Table 4**). Among 27 T0 plants, we identified 12 mutants heterozygous for *TaMLO-A1*, 8 mutants heterozygous for *TaMLO-D1*, 1 mutant heterozygous for *TaMLO-B1* but homozygous for *TaMLO-A1*, 3 mutants heterozygous for both *TaMLO-A1* and *TaMLO-D1,* and 1 mutant heterozygous for all three homoeoalleles (**Fig. 1b**, **Table 1**, **Supplementary Fig. 2** and **Supplementary Table 4**). We also found two T0 plants (T0-6 and T0-9) in which multiple types of deletions occurred at a single target site; that is, four mutation patterns were found in plant T0-6 in *TaMLO-A1* and three in T0-9 in T*aMLO-D1* (**Fig. 1c** and **Table 1**). This phenomenon has also been reported in diploid *Arabidopsis*[14] and rice[25]. The above-mentioned results support that TALENs can efficiently create targeted mutations in the wheat genome.

To investigate whether the mutations could be transmitted to the next generation, we self-pollinated nine of the T0 plants that carried mutations in the three diploid genomes, and genotyped individual T1 progeny using *MLO* allele-specific primers. For mutations that were homozygous in the T0 generation, transmission rates were 100%, and most of the mutations that were heterozygous in the T0 generation segregated in a Mendelian fashion (homozygous/heterozygous/wild type = 1:2:1) in the T1 generation (**Table 1**). For example, in plant T0-8, a mutation in *TaMLO-A1* that was homozygous in T0 was present in all 58 T1 progeny, whereas a mutation in *TaMLO-B1* that was heterozygous in T0 segregated in a 1:2:1 ratio in 58 T1 progeny (**Table 1**). However, the segregation patterns of the complex mutations found in plants T0-6 and T0-9 did not fit a Mendelian ratio, probably because these mutations were restricted to somatic cells that did not participate in the production of gametes. Notably, some new

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Table 1 Molecular and genetic analysis of TALEN-induced mutations in *TaMLO* homoeologs and their transmission to the T1 generation

Analysis of T0 plants			Mutation segregation in T1 population					
Plant ID	Genotype of *TaMLO* homoeologs	Mutation detected (bp)[a]	No. of tested plants	Wild type	Hetero	Homo	Mutation transmission (%)[b]	TALEN-free (%)[c]
T0-2	Aa	–3	56	11 (AA)	31 (Aa)	14 (aa)	80.4[d]	5.4
	BB			54 (BB)	2 (Bb)	0 (bb)		0
	DD			53 (DD)	3 (Dd)	0 (dd)		0
T0-3	Aa	–32	3	1 (AA)	1 (Aa)	1 (aa)	66.7	0
	Bb	+141		0 (BB)	3 (Bb)	0 (bb)	100	
	Dd	–11/+81		0 (DD)	0 (Dd)	3 (dd)	100	
T0-4	AA		123	114 (AA)	9 (Aa)	0 (aa)		0
	BB			121 (BB)	2 (Bb)	0 (bb)		0
	Dd	–5		30 (DD)	73 (Dd)	20 (dd)	75.6[d]	8.1
T0-5	Dd	–5	149	25 (DD)	95 (Dd)	29 (dd)	83.2[d]	6.0
T0-6	Aa	–2, –4, –3, –6	68	58 (AA)	10 (Aa)	0 (aa)	14.7	1.5
T0-7	AA		48	47 (AA)	1 (Aa)	0 (aa)		0
	BB			46 (BB)	2 (Bb)	0 (bb)		0
	Dd	–2		4 (DD)	36 (Dd)	8 (dd)	91.7	8.3
T0-8	aa	–3, –7	58	0 (AA)	0 (Aa)	58 (aa)	100	12.1
	Bb	–2/+113		16 (BB)	31 (Bb)	11 (bb)	72.4[d]	
T0-9	Aa	–10	59	19 (AA)	25 (Aa)	15 (aa)	67.8[d]	6.8
	Dd	–2, –4, –5		54 (DD)	5 (Dd)	0 (dd)	8.5	
T0-11	Aa	–3/+61	88	23 (AA)	44 (Aa)	21 (aa)	73.9[d]	8.0
	Dd	–29		21 (DD)	41 (Dd)	26 (dd)	76.1[d]	

Hetero, heterozygous; Homo, homozygous.
[a]"–" indicates deletion of the indicated number of nucleotides; "+" indicates insertion of indicated number of nucleotides; "–/+" indicates simultaneous deletion and insertion of the indicated number of nucleotide at the same site; "–,..." indicates multiple types of deletions occurring in different mutation events at the same target site. [b]Based on the number of plants carrying the observed mutation over the total number of plants tested. [c]Absence of intact TALEN construct and herbicide-resistance gene, based on the number of mutant plants not harboring the maize *Ubiquitin 1* promoter over the total number of plants tested. [d]Segregation of the heterozygous lines conforms to a Mendelian 1:2:1 ratio according to the χ^2 test ($P > 0.5$).

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mutations were detected in the T1 plants. For instance, whereas the mutation that arose in plant T0-4 was only detected in *TaMLO-D1*, the identification of additional mutations in *TaMLO-A1* and *TaMLO-B1* in its T1 progeny (**Table 1**) suggests that the TALENs remained active in the T0 and/or T1 plants. As expected, T-MLO was present in all these T1 plants (**Table 1**). We analyzed further the transmission of homozygous and heterozygous mutations from six T1 plants to their T2 offspring. Again, the homozygous mutations were 100% transmitted, whereas the heterozygous mutations segregated in a Mendelian fashion (**Supplementary Table 5**). These results demonstrate that TALEN-mediated gene modifications observed in primary transformed bread wheat plants (T0) can be stably transmitted to subsequent generations.

To investigate the possibility of achieving targeted modifications without incorporating foreign DNA into the bread wheat genome, we designed PCR primers specific for the maize *Ubiquitin 1* promoter driving the *bar* gene in plasmid pAHC20 and the TALEN gene in the T-MLO plasmid. The PCR assay failed to detect the maize

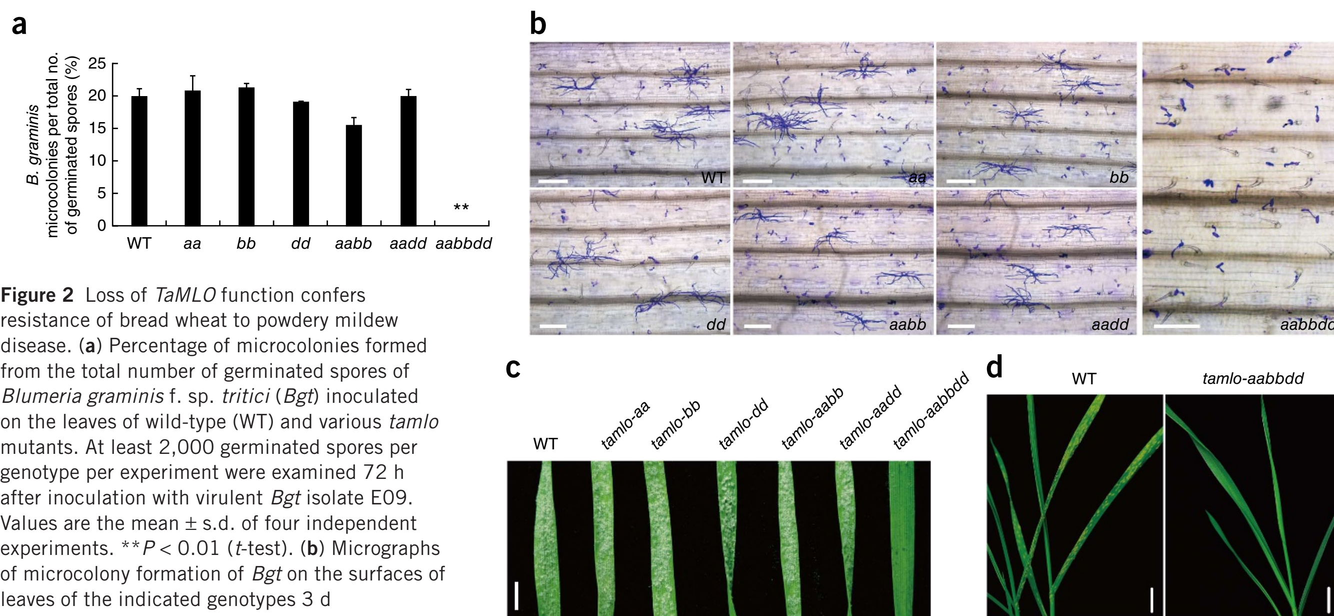

Figure 2 Loss of *TaMLO* function confers resistance of bread wheat to powdery mildew disease. (**a**) Percentage of microcolonies formed from the total number of germinated spores of *Blumeria graminis* f. sp. *tritici* (*Bgt*) inoculated on the leaves of wild-type (WT) and various *tamlo* mutants. At least 2,000 germinated spores per genotype per experiment were examined 72 h after inoculation with virulent *Bgt* isolate E09. Values are the mean ± s.d. of four independent experiments. $**P < 0.01$ (*t*-test). (**b**) Micrographs of microcolony formation of *Bgt* on the surfaces of leaves of the indicated genotypes 3 d postinoculation. Powdery mildew spores and colonies were stained with Coomassie blue. Scale bars, 200 μm. (**c**) Macroscopic infection phenotypes of representative leaves of WT and the indicated *mlo* mutants 7 d after inoculation of detached leaves with *Bgt*. Scale bar, 1 cm. (**d**) Disease symptoms of wild-type (WT) and *tamlo-aabbdd* mutant plants. The photograph was taken 7 d after inoculation *in planta*. Scale bars, 2 cm.

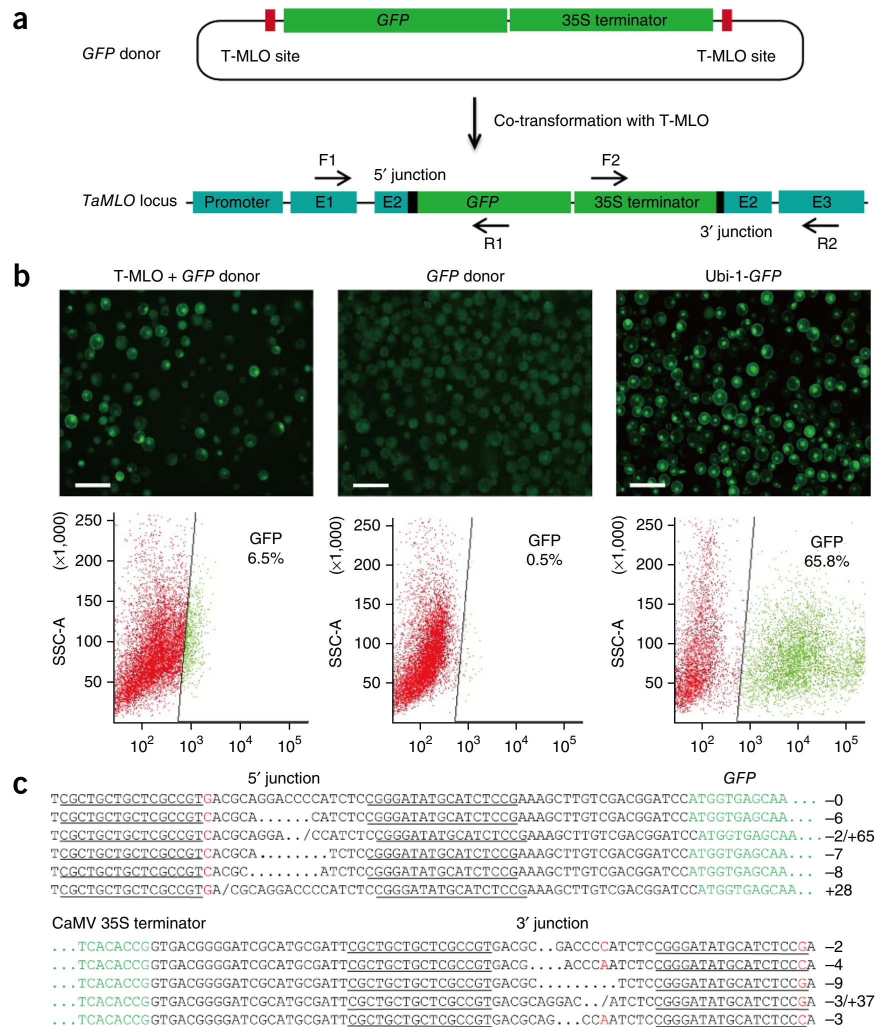

Figure 3 NHEJ-mediated knock-in of a *GFP* reporter gene at a *TaMLO* site in wheat protoplasts. (**a**) Structure of the *GFP* donor plasmid and the anticipated outcome of a *GFP* knock-in event. A cauliflower mosaic virus (CaMV) 35S terminator lies downstream of the *GFP* coding sequence. The cassette is flanked by two T-MLO sites, which generate a linear structure by recombination with the co-transformed T-MLO plasmid. The locations and names of the primers used for PCR analysis of knock-in events are shown. (**b**) Measurement of *GFP* knock-in efficiency in wheat protoplasts by flow cytometry. Three fields of protoplasts are shown. Protoplasts were transformed with the following DNA constructs (from left to right): (i) T-MLO plus *GFP* donor plasmids; (ii) *GFP* donor plasmid alone; (iii) positive control with GFP expression driven by the maize *Ubiquitin 1* promoter. Flow cytometry was used to quantify the percentage of GFP-expressing protoplasts. Scale bars, 100 μm. (**c**) Sequencing of 5′ and 3′ junctions confirm NHEJ-mediated knock-in events. The 5′ junction sequences were PCR-amplified with primers F1 and R1, and the 3′ junctions with primers F2 and R2. T-MLO sites are underlined. Inherent SNPs in the T-MLO site are highlighted in red. Sequences from the coding region of GFP and the CaMV 35S terminator are highlighted in green. The numbers on the right show the type of mutation and how many nucleotides are involved, with "–" and "+" indicating nucleotide deletion and insertion, respectively.

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Ubiquitin 1 promoter in 45 out of 652 (6.9%) T1 plants derived from 9 T0 lines and 22 out of 105 (21.0%) T2 plants derived from 3 T1 lines (**Table 1** and **Supplementary Table 5**). Two TALEN-free *tamlo-aabbdd* homozygous mutant plants were obtained by two generations of selfing of a T0-3 plant (**Supplementary Table 5**). This indicates that a TALEN-free plant carrying only the desired DNA sequence change can be obtained through genetic segregation.

We further demonstrated the application of SSNs in bread wheat by using the CRISPR-Cas9 system to generate plants mutated in a single *TaMLO* allele. Previously, we reported that the CRISPR-Cas9 system could induce sequence-specific genome modifications of *MLO* genes in wheat protoplasts[17]. Here we used the T7 endonuclease I (T7E1) assay[26] to identify mutations induced by sgMLO-A1 in wheat protoplasts and transgenic wheat plants (**Fig. 1a**,**d**,**e**, **Supplementary Fig. 3** and **Supplementary Table 1**). Although we have yet to cover CRISPR-Cas9 lines mutated in all three *TaMLO* alleles, a screen of 72 T0 transgenic wheat lines identified four independent mutants, each carrying different mutations in the *TaMLO-A1* allele (**Fig. 1d**,**e**). This mutation frequency (5.6%) is similar to that obtained using TALENs (**Supplementary Table 3**).

We assessed the impact of TALEN-induced mutations of *TaMLO* on wheat resistance to powdery mildew. Except for *tamlo-bbdd*, all of the combinations of the *TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1* homozygous mutants (*tamlo-aa*, *tamlo-bb*, *tamlo-dd*, *tamlo-aabb*, *tamlo-aadd* and *tamlo-aabbdd*) were obtained by selfing, and were genotyped by RCR-RE and sequencing (**Supplementary Fig. 4**). Seedling leaves of these mutants were challenged with conidiospores of a virulent *Bgt* race. Microscopic examination showed that the number of mildew microcolonies formed on the leaves was significantly reduced ($P < 0.01$) only in *tamlo-aabbdd* mutant plants (**Fig. 2a**,**b**). Consistent with this finding, no apparent fungal growth was observed on the leaves of the *tamlo-aabbdd* plants, although abundant fungal growth was found on the leaves of wild-type plants and those of the other mutant combinations (**Fig. 2c**,**d**). The *tamlo-aabbdd* plants also showed strong resistance to two additional virulent *Bgt* races tested (**Supplementary Fig. 5**). These results suggest that *TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1* all contribute to the response of bread wheat to *Bgt* infection, and that simultaneous mutation of the three homoeoalleles confers broad-spectrum resistance to powdery mildew. The race-specific resistance that is commonly used to develop resistant wheat varieties is controlled by a resistance (*R*) gene, and tends to break down as new *Bgt* races emerge in the field[27]. In contrast, the resistance to powdery mildew conferred by a loss-of-function *mlo* mutation has yet to be broken since its introgression into elite barley varieties three decades ago[19]. Therefore, the *tamlo-aabbdd* alleles we generated in the elite wheat cultivars might provide excellent starting materials for breeding durable and broad-spectrum resistance in bread wheat.

The important role of *TaMLO* genes in regulating powdery mildew disease resistance as described above prompted us to test if we could use NHEJ at double-strand breaks caused by TALENs to add genes downstream of *TaMLO* promoters. This might be essential in efforts to further improve the efficacy of *TaMLO* gene products in

preventing powdery mildew. We constructed a donor vector that contains a promoter-less *GFP* coding sequence and CaMV 35S terminator, flanked by T-MLO recognition sites (**Fig. 3a** and **Supplementary Fig. 6**) and co-transformed the T-MLO plasmid and the *GFP* donor vector into wheat protoplasts. Correct insertion of the *GFP* coding sequence into *TaMLO* loci rendered the protoplasts fluorescent (**Fig. 3b**). We reliably detected more fluorescent protoplasts from transformations with the TALENs and the *GFP* donor than with the *GFP* donor alone (**Fig. 3b**). Sequencing of PCR products of the genomic DNA of the transformed protoplasts confirmed that the *GFP* cassette had been inserted into the *TaMLO* loci, accompanied by small deletions and insertions due to NHEJ at the 5′ and 3′ junctions (**Fig. 3c**). We also tested targeted knock-in at the *TaMLO* loci with ssDNAs (ssDNA-1 and ssDNA-2) encoding His-tag and Myc-tag peptides, respectively (**Supplementary Fig. 7a** and **Supplementary Table 6**). The T-MLO plasmid and pAHC20, in combination with either ssDNA-1 or ssDNA-2, were co-transformed into immature wheat embryos via particle bombardment. Whereas the His-tag sequence was integrated in the correct orientation into the *TaMLO-A1* target site in one of 69 regenerated transgenic plants, in one of 39 transgenic plants the Myc-tag sequence was integrated into *TaMLO-B1* in the opposite orientation with two copies (**Supplementary Table 3** and **Supplementary Fig. 7b**,**c**). Analysis of T1 populations showed that the insertions were inherited in a Mendelian fashion (**Supplementary Fig. 7d**,**e**). These results demonstrate the feasibility of using NHEJ to target gene insertion in bread wheat for further manipulation of *TaMLO* and other genes that control important agronomic traits.

Crop improvement requires the constant creation and use of new allelic variants. The great promise of genome editing for crop improvement has only recently begun to be realized, and has only been demonstrated in very few cases[12]. Our study proves that TALENs and the CRISPR-Cas9 system can be used to generate beneficial genetic traits in hexaploid bread wheat. In addition, we show that the NHEJ pathway enables targeted DNA insertion. The latter strategy should be valuable for creating traits that cannot be engineered by simple mutagenesis. Our work presents a successful example of the use of SSNs for molecular breeding of bread wheat. The rapidity and precision with which changes can be achieved by this approach will help to improve wheat at a rate sufficient to guarantee global food security.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank D. Wang for critical reading of the manuscript and providing the E22 and B13 strains, Q. Shen for providing E09 strain, and T. Li for technical support in flow cytometry (all three are at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). This work was supported by grants from the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB11030500) and the National Basic Research Program of China (973 Program, 2011CB100702) to J.-L.Q. and a grant to C.G. from the National Natural Science Foundation of China (31271795).

AUTHOR CONTRIBUTIONS

J.-L.Q., C.G. and Y.W. designed the experiments; Y.W., X.C., Q.S., Y.Z. and J.L. performed the experiments; and J.-L.Q., C.G. and Y.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

TALEN design and construction. TALEN target sites were designed using the TAL Effector-Nucleotide Targeter 2.0 (TALE-NT) program (https://tale-nt.cac.cornell.edu/). All the target sites had a T at the –1 position, and the corresponding TAL effector arrays were constructed using the Golden Gate method[22]. Information on all the TAL effecter arrays and target sites is given in **Supplementary Table 1**. TALENs were assembled in vectors with a truncated N152/C63 backbone architecture (pZHY500 and pZHY501)[28]. The Gateway-compatible entry plasmid, pZHY013, was used as the intermediate vector to create TALEN expression vectors[28]. This plasmid contains two heterodimeric FokI nuclease domains separated by a T2A translational skipping sequence. TAL arrays in the plasmids pZHY500 and pZHY501 were released by digestion with XbaI/BamHI and subcloned into pZHY013 one by one[23,28]. One array (left array) was first cloned into pZHY013 as an XbaI/BamHI fragment; the other (right array) was then cloned into the NheI/BglII sites, which have ends compatible with XbaI and BamHI. A Gateway LR reaction was performed to clone the TALEN coding sequences into the destination vector, pYP010 (a derivative of pZHY051, ref. 28) by replacing the CaMV 35S promoter with the maize *Ubiquitin 1* promoter.

Construction of Cas9 and sgRNA expression vectors. The plasmid pJIT163 (ref. 29) was used to construct the Cas9 expression plasmid. It was digested with KpnI and HindIII and fused with the maize Ubiquitin 1 promoter to construct vector pJIT163-Ubi. Full-length *Cas9* (plant codon–optimized) products were digested with BamHI and MfeI and inserted into plasmid pJIT163-Ubi between the BamHI and MfeI sites to yield the expression vector pJIT163-Ubi-Cas9. The wheat U6 promoters and wheat gRNA scaffolds were synthesized by GenScript and cloned into pEASY-blunt vector (TransGen Biotech) to generate the pU6-gRNA plasmid. The sequences of Cas9 and the gRNAs are given in a previous publication[17]. Wheat genomic DNA region immediately preceding a 5′-NGG protospacer-adjacent motif (PAM), such as 5′-G-N(20)-GG-3′ or 5′-N(21)-GG-3′, was selected as target.

Wheat protoplast transformation. Wheat protoplasts were isolated and transformed as previously described[17]. Average transformation efficiencies were 60–80%. Protoplast transformation was carried out with 20 µg of TALEN plasmid per transformation, or a mixture of 10 µg pJIT163-Ubi-Cas9 plasmid and 10 µg pU6-gRNA plasmid.

Biolistic transformation of wheat. Biolistic transformation was performed using a PDS1000/He particle bombardment system (Bio-Rad) with a target distance of 6.0 cm from the stopping plate at helium pressure 1,100 psi as previously described[30]. Plasmid DNAs (T-MLO and pAHC20) were mixed in a 1:1 (1:1:1 for Cas9, gRNA and pAHC20) molar ratio prior to bombardment. After bombardment, embryos were transferred to callus induction medium. In the third or fourth week, all calli were transferred to selective regeneration medium containing 5 mg/l phosphinothricin (PPT). PPT was present in all subsequent tissue culture procedures including 2 rounds of regeneration (4 weeks) and 2 rounds of rooting (4 weeks). After 10–12 weeks, T0 transgenic plants were obtained, transferred into soil and grown in a greenhouse.

Screening of SSN-induced mutations. Genomic DNA from individual wheat plants was extracted using the high-throughput Automation Workstation Biomek FX (Beckman Coulter) with the magnetic bead–based DNA extraction kit (GeneOn Biotech). The PCR-RE digestion screen assay and T7E1 (ViewSolid Biotech) assay were used to identify the mutations[23,26]. The PCR products amplified with *TaMLO*-specific primers (**Supplementary Table 2**) from individual mutant plants were cloned into pUC-T vector (CWBIO) for sequencing. Mutation frequencies (indels (%)) in protoplasts were calculated by measuring band intensities with UVP VisionWorks LS Image Acquisition Analysis Software 7.0 (ref. 17).

Powdery mildew infection and microscopic analyses. Wheat plants were grown on soil in controlled environment chambers at 22°C and 16-h photoperiod with light intensity ranging from 400 to 1,000 µmol m^{-2} s^{-1}. Powdery mildew infection and microscopic analyses were performed as previously reported[31] with some modifications. Leaves originating from the main stem (leaves 2, 3, and 4) were cut into 5 cm segments and immediately placed in Petri dishes containing 1% (w/v) distilled water agar and 8.5 mM benzimidazole. The leaf segments were incubated at 22°C in continuous light (100 µmol m^{-2} s^{-1}) for 4 h, then inoculated with virulent strains of *Blumeria graminis* f. sp. *tritici* (*Bgt*) E09, E22 or B13 to give approximately 15 to 20 sporulating colonies per cm^2 and incubated at 22°C in continuous light (100 umol m^{-2} s^{-1}). Seventy-two hours after inoculation, the leaf segments were fixed with 1:1 (v/v) ethanol/ acetic acid for 24 h, cleared with lactoglycerol (1:1:1 (v/v) lactic acid/glycerol/H_2O) for 48 h, and stained for 7 sec with Coomassie blue (0.6% (w/v) Coomassie Brilliant Blue R 250 (Sigma; in methanol) to visualize the fungal structure, finally rinsed in distilled water and mounted in 50% (v/v) glycerol prior to microscopy. Samples were observed and analyzed under an Olympus BX51 light microscope, and photographs were taken using software Cellsens Entry 1.21.

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doi:10.1038/nbt.2969