the plant journal

SEB Society for Experimental Biology

The Plant Journal (2017) **91,** 714–724 doi: 10.1111/tpj.13599

Simultaneous modification of three homoeologs of *TaEDR1* by genome editing enhances powdery mildew resistance in wheat

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Received 9 February 2017; revised 23 April 2017; accepted 3 May 2017; published online 14 May 2017.

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SUMMARY

Wheat (*Triticum aestivum* L.) incurs significant yield losses from powdery mildew, a major fungal disease caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*). enhanced disease resistance1 (EDR1) plays a negative role in the defense response against powdery mildew in *Arabidopsis thaliana*; however, the *edr1* mutant does not show constitutively activated defense responses. This makes *EDR1* an ideal target for approaches using new genome-editing tools to improve resistance to powdery mildew. We cloned *TaEDR1* from hexaploid wheat and found high similarity among the three homoeologs of *EDR1*. Knock-down of *TaEDR1* by virus-induced gene silencing or RNA interference enhanced resistance to powdery mildew, indicating that *TaEDR1* negatively regulates powdery mildew resistance in wheat. We used CRISPR/Cas9 technology to generate *Taedr1* wheat plants by simultaneous modification of the three homoeologs of wheat *EDR1*. No off-target mutations were detected in the *Taedr1* mutant plants. The *Taedr1* plants were resistant to powdery mildew and did not show mildew-induced cell death. Our study represents the successful generation of a potentially valuable trait using genome-editing technology in wheat and provides germplasm for disease resistance breeding.

Keywords: genome editing, CRISPR/Cas9, hexaploid wheat, *Triticum aestivum* L., powdery mildew, *Blumeria graminis*, EDR1.

INTRODUCTION

Bread wheat (*Triticum aestivum* L. 2*n* = 42; AABBDD) is a major food crop worldwide (Hoisington *et al.*, 1999). Powdery mildew, caused by the biotrophic fungal pathogen powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*), is one of the most important diseases limiting bread wheat production (Singh *et al.*, 2016). Control of powdery mildew in wheat is primarily achieved by the application of fungicide and the use of germplasm with disease resistance genes. Unfortunately, the disease resistance genes only confer resistance to specific powdery mildew isolates. To date, 78 powdery mildew resistance alleles have been identified and 50 loci have been designated. Many loci have been used in wheat breeding, but only four genes, including *Pm2*, *Pm3*, *Pm8* and *Pm21,* have been molecularly characterized or cloned (Yahiaoui *et al.*, 2004; Cao *et al.*, 2011; Hurni *et al.*, 2013; Sanchez-Martin *et al.*, 2016). The race-specific nature of these genes is not ideal, since virulent pathogen mutants of *Bgt* can escape recognition and rapidly overcome this resistance. Additionally, the environmental impact of fungicide use and the potential

for the development of fungicide tolerance by the pathogen necessitates the development of other control options. Identifying and exploiting genes that function in basal defense may have high potential for reducing yield losses caused by *Bgt*.

Hexaploid wheat is particularly difficult for forward and reverse genetic analyses: it has high ploidy, a large genome (17 Gb) and a high content of repetitive sequences, including transposable elements (80–90%) (Slade *et al.*, 2005). These attributes limit the feasibility of some gene modification methods, such as virus-induced gene silencing (VIGS) and RNA interference (RNAi), which have some utility in the analysis of gene function, but do not produce heritable or stable modifications (Unniyampurath *et al.*, 2016). In contrast, genome editing is precise, widely applicable and has high mutation efficiency, making it an ideal tool for genome modification and the analysis of gene function. In addition, mutation(s) induced by genome editing are generally stably inherited (Symington and Gautier, 2011; Voytas, 2013). Genome editing techniques have been successfully used in a variety of crops, including maize (Liang *et al.*, 2014; Svitashev *et al.*, 2016; Kelliher *et al.*, 2017), rice (Shan *et al.*, 2013a,b, 2015; Zhou *et al.*, 2014; Li *et al.*, 2016), wheat (Shan *et al.*, 2013b; Upadhyay *et al.*, 2013; Wang *et al.*, 2014; Zhang *et al.*, 2016), barley (Lawrenson *et al.*, 2015), soybeans (Tang *et al.*, 2016), tomato (Soyk *et al.*, 2017), tobacco (Xie *et al.*, 2017) and citrus (Jia *et al.*, 2017).

Mutations in the mildew resistance locus (*MLO*) in barley lead to broad-spectrum and durable resistance to barley powdery mildew (Wolter *et al.*, 1993; Humphry *et al.*, 2006), but the barley *mlo* mutant shows pleiotropic phenotypes, including spontaneous cell death and early senescence (Wolter *et al.*, 1993; Peterhansel *et al.*, 1997). MLO is highly conserved in plant species (Kusch *et al.*, 2016). We previously showed that transcription activator-like effector nuclease (TALEN)-induced mutation of all three *TaMLO* homoeologs confers heritable broad-spectrum resistance to powdery mildew, but the TALEN-based *Tamlo* knock-out plants showed leaf chlorosis (Wang *et al.*, 2014; Acevedo-Garcia *et al.*, 2016). Acevedo-Garcia *et al.* (2016) recently used targeting-induced local lesion in genomes (TILLING) technology to generate wheat *mlo* mutant lines, which display enhanced resistance to powdery mildew. These TILLING-derived mutants contain only non-conservative mutations in *TaMLO* and did not show obvious pleiotropic phenotypes (Acevedo-Garcia *et al.*, 2016).

In Arabidopsis, enhanced disease resistance 1 (EDR1), a Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK) plays a negative role in powdery mildew resistance (Frye *et al.*, 2001). Powdery mildew resistance in *edr1* is accompanied by mildew-induced mesophyll cell death, marginal growth reduction and the accumulation of callose (Frye and Innes, 1998). Other autoimmune mutants, such as *cpr1* (mutant of *CONSTITUTIVE EXPRESSER OF PR GENES 1*), however, have much higher reductions in growth than *edr1* mutants (van Hulten *et al.*, 2006). These *edr1*-associated resistance phenotypes are dependent on the salicylic acid (SA) pathway, but are independent of ethylene and the jasmonic acid pathway (Frye *et al.*, 2001; Tang *et al.*, 2005). EDR1 could be recruited by EDR4 to the fungal penetration site via physical interaction (Wu *et al.*, 2015). In addition, EDR1 interacts with MKK4/MKK5 and negatively affects the protein levels and kinase activity of MPK3 and MPK6 to fine-tune innate plant immunity (Zhao *et al.*, 2014). EDR1 also negatively regulates arabidopsis toxicos en levadura1 (ATL1), a positive regulator of cell death, to affect plant immunity (Serrano *et al.*, 2014).

EDR1 is highly conserved across plant species (Frye *et al.*, 2001), and in rice *OsEDR1* also plays negative roles in the defense response. The expression of *OsEDR1* could be induced by jasmonic acid (JA), SA, ethylene, abscisic acid and the fungal elicitor chitosan (Kim *et al.*, 2003). The RNAi plants or T-DNA insertion mutants of *OsEDR1* showed enhanced resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In the rice–*Xoo* interaction, *Os*EDR1 promotes the synthesis of ethylene but suppresses SA- and JA-associated defense signaling (Shen *et al.*, 2011).

We reasoned that EDR1 could be a good candidate for improving resistance to powdery mildew in wheat since the *edr1* mutation only slightly affects growth in Arabidopsis. In this study, we cloned *TaEDR1* in hexaploid wheat and showed that knock-down *TaEDR1* mutants from VIGS or RNAi increased resistance to virulent *Bgt* isolates. Using clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) technology we then generated wheat *edr1* plants by simultaneous modification of the three homoeologs of TaEDR1. The *Taedr1* plants did not exhibit mildew-induced cell death and will be useful for wheat breeding programs.

RESULTS

Isolation and analysis of mutant alleles of the *TaEDR1* homoeologs

Rapid-amplification of cDNA ends, homoeolog cloning, and sequencing were used to identify three highly conserved homoeologs of *TaEDR1* from Kenong 199 (KN199), an elite semi-winter bread wheat variety susceptible to powdery mildew. These three homoeologs of *TaEDR1* encoded a protein with a typical EDR1 N-terminal regulatory domain and a kinase domain. The coding sequences of the three copies share similarities of 99.14% and encode proteins of 959, 963 and 962 amino acids, respectively, with 98.48% similarity (Figure 1). We performed BLAST search with the *TaEDR1* gene on the Ensembl plants website (http://plants.ensembl.org/Triticum_aestivum/Tools/Bla

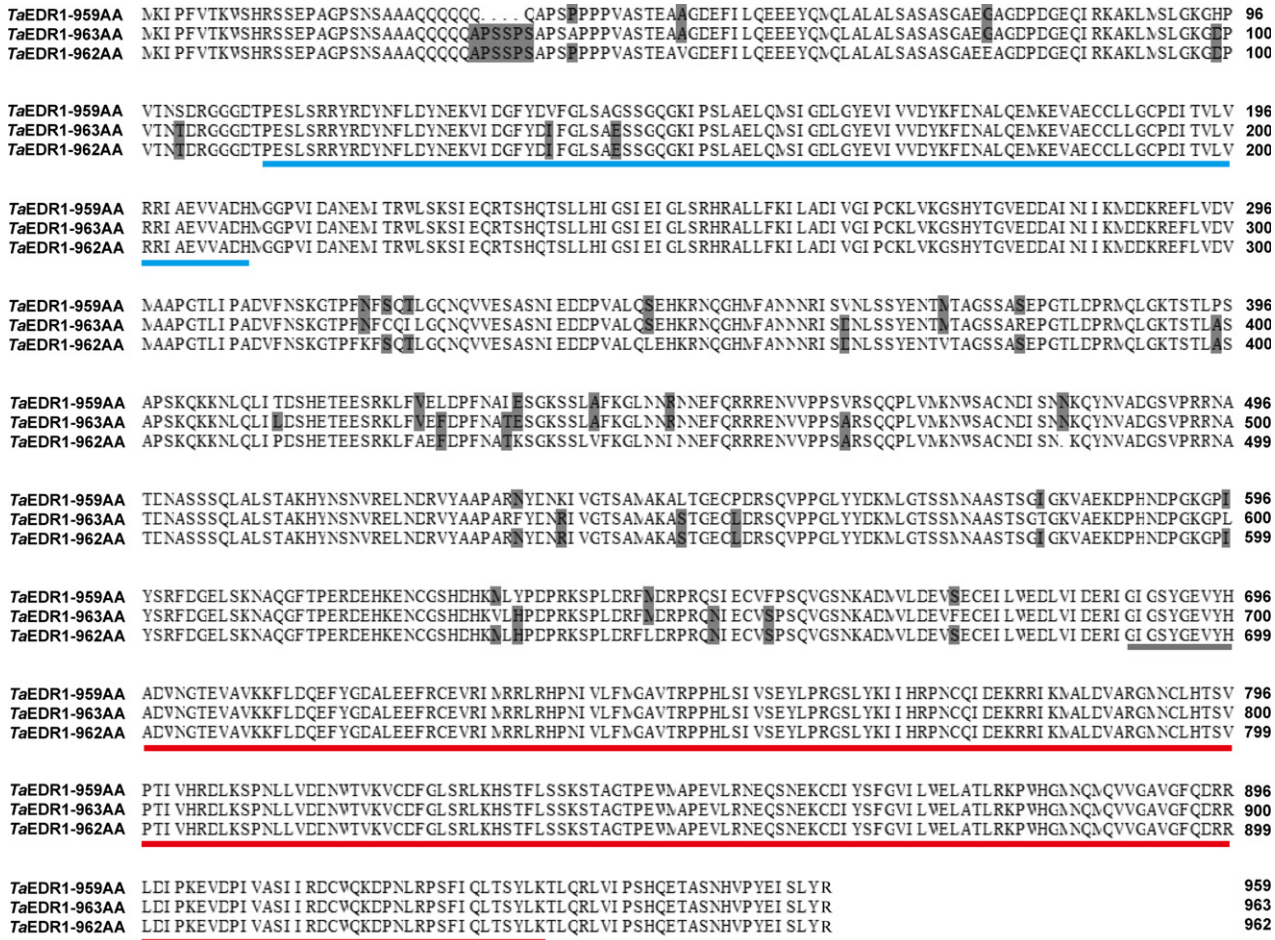

Figure 1. Homology-based identification of the bread wheat EDR1 (TaEDR1) homoeologs.
TaEDR1-1A (TaEDR1-959AA), TaEDR1-1B (TaEDR1-963AA), and TaEDR1-1D (TaEDR1-962AA) represent the amino acid sequences of the three homoeologs of TaEDR1. The EDR1 N-terminal regulatory domain and kinase domain are underlined separately in blue and red, respectively. Numbers on the right indicate the serial orders of sequences. Polymorphic amino acids in TaEDR1 are shaded in gray.

st?db=core) and found no additional EDR like genes in the wheat genome.

We next used length polymorphisms in the second exon of the *TaEDR1* genome sequence and Chinese Spring nullisomic–tetrasomic lines to localize three copies of *TaEDR1* on chromosomes 1A, 1B and 1D, respectively (Figure S1 in the online Supporting Information). These results were further confirmed using sequences of *TaEDR1* as queries to perform a BLAST search of the chromosome-based draft sequence of hexaploid wheat. The three homoeologs of *TaEDR1* were found on chromosomes 1AS (959AA), 1BL (963AA) and 1DL (962AA). Thus, we designated them *TaEDR1-1A*, *TaEDR1-1B* and *TaEDR1-1D*, respectively.

TaEDR1 is not considered a major disease resistance gene, but may be involved in basal defense. This is suggested by indistinguishable coding sequences of *TaEDR1* homoeoalleles among susceptible (KN199, Chinese Spring, Xiaoyan 54) and resistant (Xiaobaidong Mai, Youbailan, Fuzhuang) varieties to *Bgt* isolate E09 (Figure S2). EDR1 was also found to be highly conserved between diploid and hexaploid wheat (Figure S3).

Phylogenetic analysis of TaEDR1 proteins from various plant species showed that the TaEDR1-1A, TaEDR1-1B and TaEDR1-1D are most closely related to barley (*Hordeum vulgare*) HvEDR1 (Figure S4), with a sequence similarity of 89.11%. Among wheat, rice and Arabidopsis, the EDR1 proteins showed a 90.19% similarity in the kinase domain, and over 60% similarity in the EDR1 N-terminal regulatory domain.

Knock-down of *TaEDR1* enhances the resistance of wheat to powdery mildew

To determine if *TaEDR1* is involved in regulating the defense response against *Bgt* in bread wheat, a VIGS strategy using the Barley stripe mosaic virus (BSMV) was used to knock down all three homoeologs of *TaEDR1* (*TaEDR1-1A*, *TaEDR1-1B* and *TaEDR1-1D*) in KN199 by targeting the sequence that encodes a highly conserved region in the

EDR1 N-terminal regulatory domain. BSMV:*PDS* was used to silence wheat phytoene desaturase (PDS) (Holzberg *et al.*, 2002; Scofield *et al.*, 2005) to visualize the occurrence of virus infection and gene silencing. The transcript levels of *TaEDR1* were examined by quantitative PCR. The relative expression of *TaEDR1* was significantly reduced in the BSMV:*TaEDR1*-treated plants compared with the BSMV:*GFP* control plants (Figure 2a), indicating that the VIGS system was effective. The VIGS leaves were inoculated with *Bgt* isolate *E09*, which is virulent to KN199, to assess the resistance. The pathogen produced fewer spores on BSMV:*TaEDR1* leaves than on the BSMV:*GFP* and BSMV:*PDS* leaves (Figure 2b).

Hydrogen peroxide (H_2O_2) and callose deposition are usually components of plant defense responses; we therefore stained the VIGS leaves with trypan blue, 3,3′-diaminobenzidine (DAB) and aniline blue to visualize the penetration of fungal-structures of *Bgt*, H_2O_2 accumulation and callose deposition under a microscope. Compared with the control, the formation of mature *Bgt* mycelium was inhibited in the BSMV:*TaEDR1* samples (Figure 2c), while the accumulation of H_2O_2 and callose deposition surrounding the fungal penetration site significantly increased (Figure 2d, e).

We also examined whether the downregulation of *TaEDR1* would affect the transcript levels of *TaPR1*, the defense-related gene that is induced by pathogens and SA. As shown in Figure 3(a), a significant induction of *TaPR1* was detected in the BSMV:*TaEDR1* samples at 5 days post-inoculation (dpi) compared with the control. Taken together, knock-down of *TaEDR1* by VIGS enhances the resistance of bread wheat to powdery mildew.

In Arabidopsis, *edr1*-mediated powdery mildew resistance requires the SA signaling pathway (Frye *et al.*, 2001). We therefore examined whether SA accumulation was altered in *TaEDR1*-silenced wheat plants. As shown in Figure 3(b), we found that the BSMV:*TaEDR1* samples accumulated significantly higher levels of free SA than control BSMV:*GFP* plants. This suggests that BSMV:*TaEDR1*-mediated resistance to powdery mildew might also depend on the SA signaling pathway.

To further investigate *TaEDR1,* we generated *TaEDR1* RNAi plants and assessed the transgenic plants for *Bgt* resistance. The sequence encoding a conserved region of 178 amino acids with 98.82% homology, just upstream of the kinase domain, was chosen to design the targets to knock down all three copies of *TaEDR1* in KN199. Twenty-two independent T_0 transgenic lines were generated with the RNAi construct that expressed double-stranded RNA for *TaEDR1* silencing. The T_1 transgenic plants were confirmed by *BAR* gene examination (Figure 4a). The RNAs of different plants (T1-8-1, T1-8-2 and T1-8-3) were mixed. *TaEDR1* expression levels were found to be significantly reduced in the RNAi plants compared with the KN199 control (Figure 4b). T_1 RNAi plants were evaluated for conidia formation following infection with *Bgt* isolate E09. Leaves of RNAi plants had significantly fewer conidia than the controls (Figure 4c). We

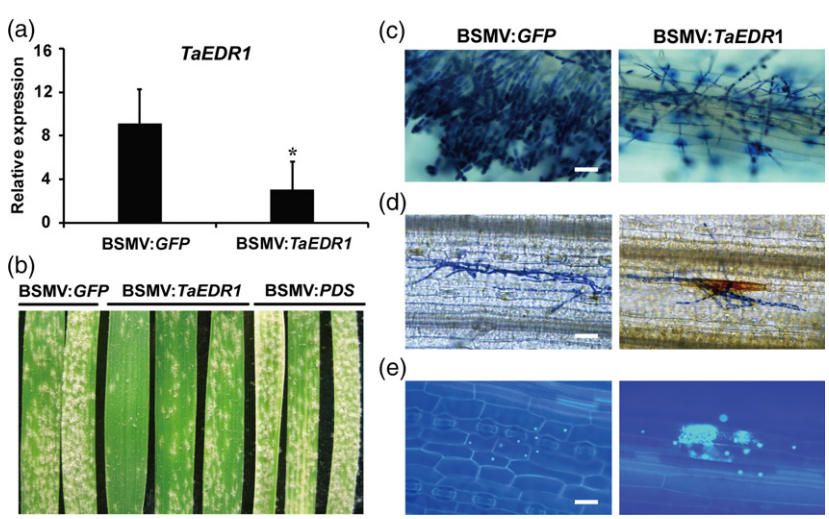

Figure 2. Silencing of *TaEDR1* enhances wheat resistance to *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolate *E09*.
(a) The transcript accumulation of *TaEDR1* in BSMV:*GFP* (control) and BSMV:*TaEDR1* plants was examined by quantitative reverse transcription PCR (qRT-PCR). *ACTIN* was used as an internal control. Error bars represent standard deviation of values obtained from at least three independent biological samples. The asterisk (*) indicates a statistically significant difference ($P < 0.05$, Student's *t*-test).
(b) Plants infected with the fungus were inoculated with *Bgt* isolate *E09* and photographs were taken at 7 days post-inoculation (dpi).
(c) Infected leaves were stained with trypan blue at 7 dpi to visualize fungal structures (blue staining). Bar = 50 μm.
(d) Infected leaves were stained with 3,3′-diamino benzidine-HCl (DAB) and trypan blue sequentially at 2 dpi to visualize hydrogen peroxide (brown staining) and fungal structures (blue staining). Bar = 50 μm.
(e) Infected leaves were stained with aniline blue at 2 dpi to visualize callose deposition (bright spots). Bar = 50 μm.

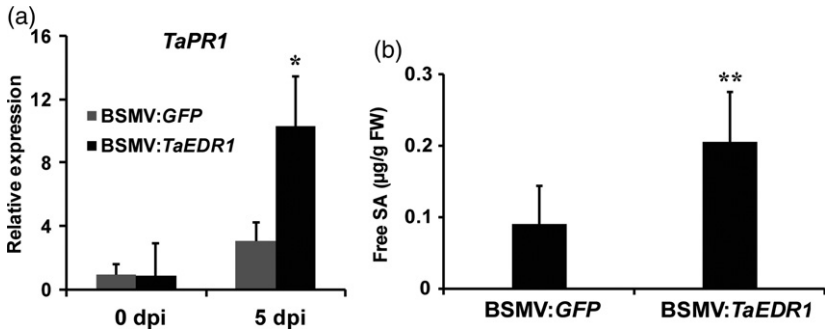

Figure 3. Silencing of *TaEDR1* increases *TaPR1* transcript accumulation and the free SA level.
(a) The relative fold change of *TaPR1* transcript accumulation was examined by qRT-PCR at the indicated time points for infected leaves. *ACTIN* was used as an internal control. Error bars represent the standard deviation of values obtained from at least three independent biological samples. The asterisk (*) indicates a statistically significant difference between Barley stripe mosaic virus (BSMV):*GFP* and BSMV:*TaEDR1* at 5 days post-inoculation (dpi) ($P < 0.05$, Student's *t*-test).
(b) The free SA level was measured in leaves of BSMV:*GFP* and BSMV:*TaEDR1* inoculated with *Blumeria graminis* f. sp. *tritici* isolate *E09* at 7 dpi. Two asterisks (**) indicate a statistically significant difference at $P < 0.01$.

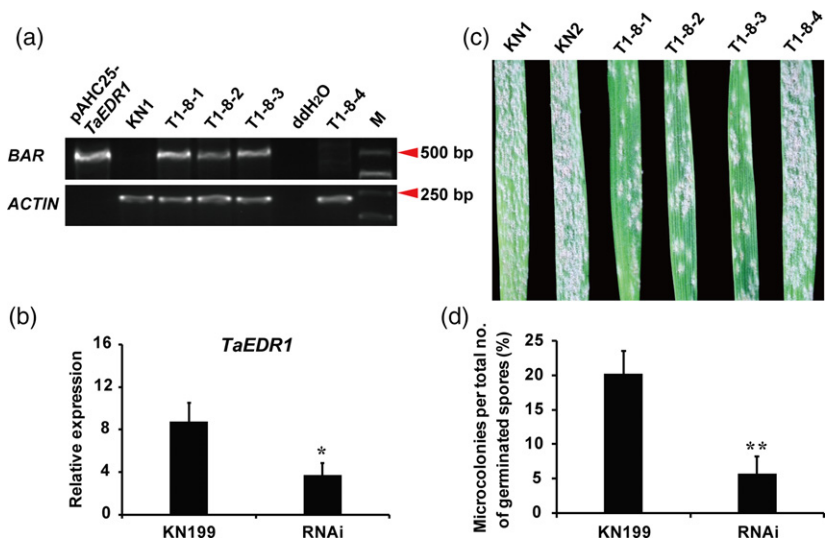

Figure 4. RNAi-mediated silencing of *TaEDR1* in the T_1 generation conferred resistance to *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolate *E09*.
(a) Transcript accumulation of the *BAR* gene as a marker of *TaEDR1* RNAi T_1 plants (T1-8-1, T1-8-2, T1-8-3 and T1-8-4) was examined by RT-PCR. *ACTIN* was used as an internal control. KN1 was used as a negative control, and pAHC25-*TaEDR1* represented the positive control. M is a marker.
(b) Transcript accumulation of *TaEDR1* of KN199 and *TaEDR1*-RNAi (RNA interference) plants was examined by qRT-PCR. *ACTIN* was used as an internal control. Error bars represent standard deviation of values obtained from at least three independent biological samples. The asterisk (*) indicates a statistically significant difference ($P < 0.05$, Student's *t*-test).
(c) The phenotypes of *TaEDR1* RNAi T_1 generation leaves inoculated with *Bgt* isolate *E09* at 7 days post-inoculation. Leaves labeled with T1-8-1, T1-8-2, T1-8-3 (resistant) and T1-8-4 (susceptible) represent different plants from the *TaEDR1* RNAi T_1 generation, and KN1 and KN2 represent control leaves from KN199.
(d) Microcolonies per total number of germinated spores (%) was used to compare *Bgt* infection levels on the KN199 control with *TaEDR1*-RNAi T_1 plants. Successfully colonized *Bgt* and spores that did not form a colony were counted separately. Means and SD were calculated with data from three independent biological replicates. Two asterisks (**) indicate a statistically significant difference at $P < 0.01$ (Student's *t*-test).

also evaluated plant susceptibility to *Bgt* isolate *E09* by calculating the micro-colony formation index (MI). KN199 had an MI of 20%, but RNAi plants (average of T1-8-1, T1-8-2 and T1-8-3) were significantly less colonized with an MI of only 5% (Figure 4d). We microscopically examined the development of powdery mildew fungus in *TaEDR1* RNAi plants and the KN199 controls. RNAi plants showed restrained development of *Bgt* E09 hyphae and mature conidia rarely developed (Figure 5a). In addition, H_2O_2 accumulation and callose deposition were highly induced near the penetration site in the leaves of RNAi plants (Figure 5b, c). The *TaPR1* expression levels also significantly increased in RNAi plants over controls (Figure 5d). We also looked at the heritability of powdery mildew resistance by inoculating T_2 *TaEDR1* RNAi plants with *Bgt* isolate E09. As shown in Figure 5(e), the improved resistance levels of plants in the T_2 generation were consistent with the T_1 generation.

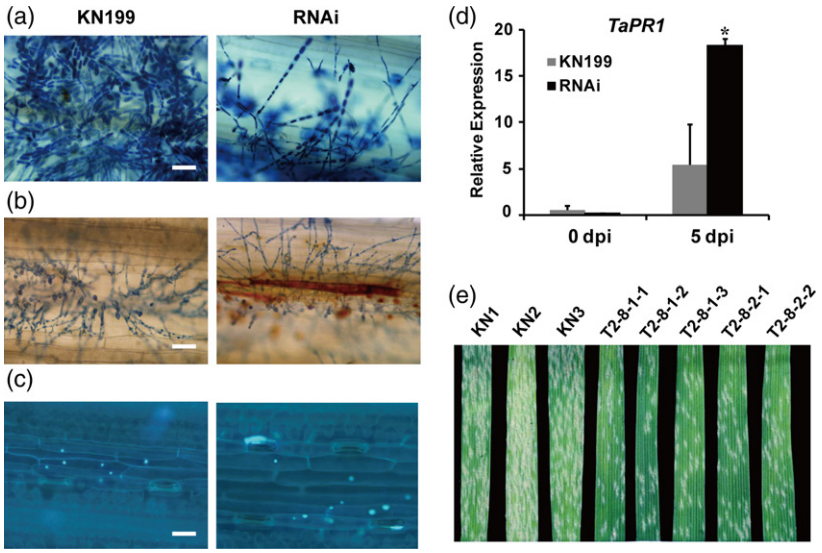

Figure 5. The RNA interference (RNAi)-mediated silencing of *TaEDR1* enhanced wheat resistance to *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolate *E09*, with evidence that resistance could be inherited in the T_2 generation.
(a) Infected leaves of T_1 generation plants were stained with trypan blue at 7 days post-inoculation (dpi) to visualize fungal structures (blue staining). KN199, control; RNAi, RNAi plants. Bar = 50 μm.
(b) Infected leaves were stained with 3,3′-diamino benzidine-HCl (DAB) and trypan blue sequentially at 4 dpi to visualize hydrogen peroxide (brown staining) and fungal structures (blue staining). Bar = 50 μm.
(c) Infected leaves were stained with aniline blue at 2 dpi to visualize callose deposition (bright spots). Bar = 50 μm.
(d) *TaPR1* transcript accumulation in KN199 and T_1 generation plants (RNAi) was examined by qRT-PCR at the indicated time points. *ACTIN* was used as an internal control. Error bars represent the standard deviation of values obtained from at least three independent biological samples. The asterisk (*) indicates a statistically significant difference ($P < 0.05$; Student's *t*-test).
(e) The phenotypes of *TaEDR1* RNAi T_2 plants inoculated with *Bgt* isolate *E09* at 7 dpi. Leaves labeled with T2-8-1-1 to T2-8-2-2 represent different *TaEDR1* RNAi plants from the T_2 generation. KN1, KN2 and KN3 represent control leaves from KN199.

Knock-out of *TaEDR1* by CRISPR/Cas9 enhances powdery mildew resistance in bread wheat

We used the CRISPR/Cas9 system (Jinek *et al.*, 2012) to further validate the function of TaEDR1 and to generate wheat *edr1* mutant plants. The single guide RNA (T-EDR1) was designed to target a highly conserved region in the fourth exon (Figure 6a). Particle bombardment of KN199 immature embryos successfully generated T_0 events. Five mutant lines (T0-1 to T0-5) were identified via PCR restriction enzyme digestion assay (PCR-RE assay). To identify the mutation types, specific primers were designed to sequence the target region of the A, B and D genomes. Sequencing analyses showed that T0-1, T0-2 and T0-3 had frameshift mutations in all three genomes; however, they were chimeric in the target sites of genomes A and B – T0-4 contained mutations in *TaEDR1-1A* and *TaEDR1-1B*, but not in *TaEDR1-1D*, and T0-5 contained a mutation only in *TaEDR1-1B* (Table S1). We analyzed 207 T_1 plants by PCR-RE and sequencing, but only identified five homozygous T_1 mutants (three from T0-2 and two from T0-3) with frameshift mutations in all three diploid genomes, (Figure 6b, c). The resistance of the T_1 mutants was tested by challenging with *Bgt* E09. As shown in Figure 6(d), the T_1 mutants with frameshift mutations in three homoeologs of *TaEDR1* had fewer mildew micro-colonies and reduced susceptibility to infection with *Bgt* E09 compared with KN199 controls (KN1, KN2). Significantly, we observed no mildew-induced cell death in this experiment. KN199 and triple *EDR1* knock-out plants were indistinguishable at tillering and jointing growth stages in the field (Figure S8).

We used CasOT (http://eendb.zfgenetics.org/casot/) to predict the potential genome-wide off-target sites for T-EDR1 based on sequence similarity. Seven potential off-target sites were identified on six chromosomes of bread wheat, with two- to four-nucleotide mismatches to the recognition site. The PCR-RE assay was performed on these seven sites, and no off-target mutations were detected in the T_1 mutants (Figure S5). To further validate these results, we sequenced the PCR products, and no mutations were found at those sites. Stable inheritance of *Taedr1* mutations to the T_1 generation was confirmed for all T_0 mutant forms with a transmission rate of 97.5–100% (Table S1). In summary, *TaEDR1* knock-out mutants in hexaploid wheat had enhanced resistance to powdery mildew and were stably transmitted to a subsequent generation.

DISCUSSION

In this study we cloned and functionally characterized EDR1 from hexaploid wheat. Three homoeologs of *EDR1* were identified and shown to have very similar sequences.

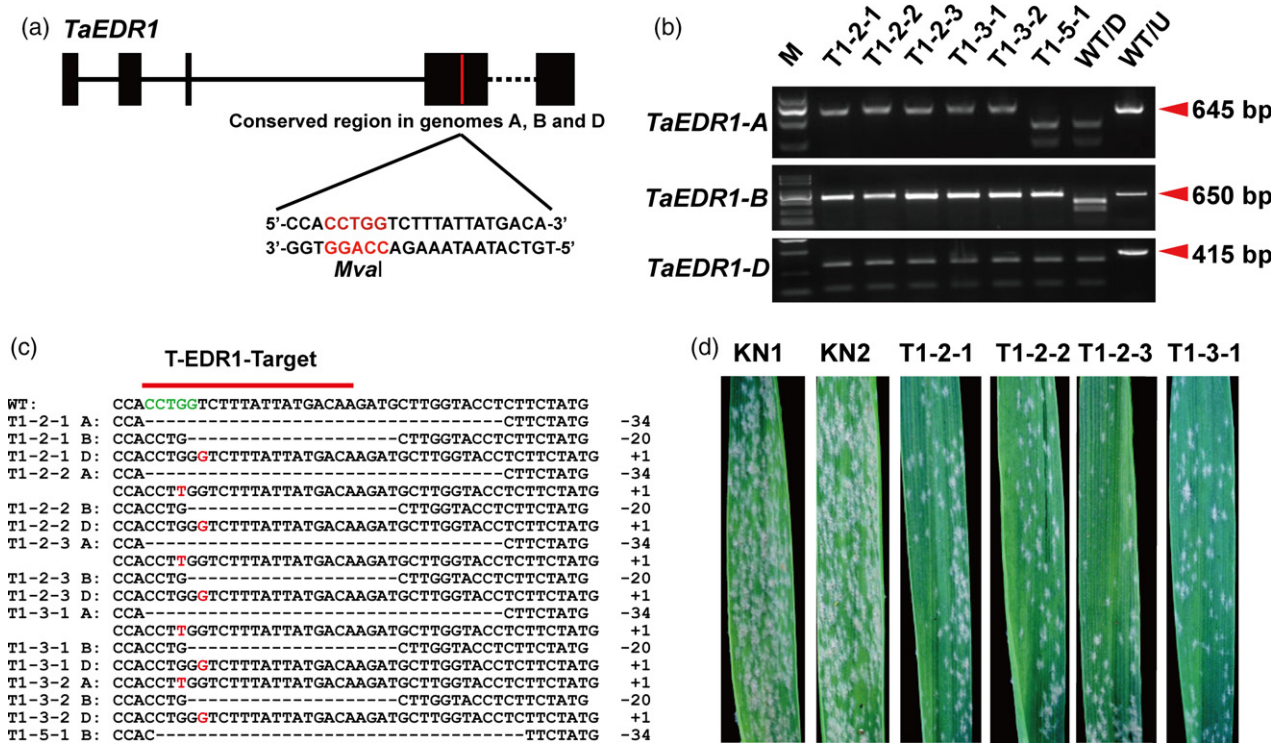

Figure 6. Knock-out of *TaEDR1* by CRISPR/Cas9 leads to enhanced resistance to *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolate *E09* in wheat.
(a) Sequence of the target site within the conserved region in the fourth exon of *TaEDR1* homoeologs. The restriction enzyme site *Mva*I, used for PCR-RE detection, is highlighted in red.
(b) The PCR-RE assays analyzing five CRISPR/Cas9-induced mutants in genomes A (TaEDR1-A), B (TaEDR1-B), and D (TaEDR1-D), respectively. Red arrowheads indicate the PCR products containing mutation sites. Lanes labeled T1-2-1 to T1-3-2 represent the five *TaEDR1* mutants. T1-5-1 represents a mutant with DNA modified only in genome B. WT/D and WT/U represent KN199 with (D) or without (U) *Mva*I digestion.
(c) Genotypes of six representative mutants were identified by sequencing. The *Mva*I restriction enzyme site in the wild type (WT) is indicated with green letters. Deleted nucleotides are represented 'by '–', and' inserted nucleotides are highlighted in red. The numbers on the right represent the number of nucleotides involved in the indel-created events with '+' or '–'.
(d) Leaf phenotypes of T1-2-1, T1-2-2, T1-2-3 and T1-3-1 compared with KN199 (KN1, KN2) at 7 dpi with *Bgt* E09.

Knock-down *TaEDR1* lines were created with VIGS or RNAi and shown to have enhanced resistance to powdery mildew. More importantly, we generated *Taedr1* plants using CRISPR-Cas9 technology and confirmed their resistance to powdery mildew. These corroborating results prove that TaEDR1 plays a negative role in powdery mildew resistance.

Although *EDR1* is well characterized in Arabidopsis, less is known about wheat *EDR1*. This gene could function differently in these diverse species, especially with the similar EDR1 homoeologs present in each of the three wheat genomes. We showed that simultaneous knock-out of all three homoeologs of *TaEDR1* confers resistance to powdery mildew. It would be interesting to examine whether these three homoeologs are functionally redundant or if they have additive effects. The *Taedr1* mutant that was generated by CRISPR/Cas9 provides the possibility to address this question. We are now crossing the *Taedr1* plants with the KN199 parental line and will generate and characterize all the respective single- or double-mutant plants. Future experiments will help differentiate the functions of the three homoeologs in resistance.

Plants need to balance immunity with growth and development. Overactive immunity could cause growth defects in plants (Belkhadir *et al.*, 2014; Lozano-Duran and Zipfel, 2015). For example, the autoimmune *cpr1* mutant in Arabidopsis displays a constitutive immune response and has significant growth defects. In contrast, the Arabidopsis *edr1* mutant shows only a marginal reduction of growth (van Hulten *et al.*, 2006). The degree to which the *edr1* mutation impacts growth is age dependent, with most effects observed only in late developmental stages (van Hulten *et al.*, 2006). In wheat, it is worth noting that *Taedr1* plants displayed only moderate resistance to powdery mildew, in contrast to the high resistance seen in knock-out *Tamlo* wheat plants (Wang *et al.*, 2014). The moderate resistance of *Taedr1* plants may have a lower fitness cost and, ultimately, more utility than the high resistance of the *Tamlo* knock-out plants (Acevedo-Garcia *et al.*, 2016). Consistent with this notion, we did not observe any obvious

defects in *Taedr1* plant growth under our conditions. This makes the *Taedr1* plants good starting materials for breeding powdery mildew resistance. We are currently assessing the performance of *Taedr1* plants under field conditions to confirm our results in the field and to better understand the fitness cost.

In Arabidopsis, *edr1*-mediated disease resistance requires the SA pathway; however, it remains to be seen whether the resistance of *Taedr1* is similarly dependent on SA. Similar to Arabidopsis *edr1* plants, knock-down of wheat *EDR1* expression by VIGS also leads to higher accumulation of SA. Interestingly, in contrast to Arabidopsis, where the powdery mildew resistance in *edr1* is associated with cell death, and rice, where *OsEDR1* RNAi plants and T-DNA insertion mutants show significant spontaneous cell death (Shen *et al.*, 2011), the *Taedr1* plants did not show any visible cell death after powdery mildew infection. This suggests that resistance in *Taedr1* is independent of cell death, and the role of EDR1 in plant species may differ in immunity, or at least in regulation of cell death.

In addition to powdery mildew resistance, Arabidopsis *edr1* plants also showed enhanced resistance to infection by bacteria and oomycetes (Pan *et al.*, 2012). In rice, the *Osedr1* plants or *OsEDR1* RNAi plants displayed enhanced resistance to the bacterial pathogen *Xanthomonas oryzae* pv *oryzae* (*Xoo*) (Shen *et al.*, 2011). These observations indicate that loss of function of EDR1 can lead to broad-spectrum resistance. Therefore, it would be interesting to examine the resistance spectrum of the *Taedr1* plants using additional wheat pathogens, such as other powdery mildew strains, *Fusarium graminearum* strains and strains of rust (*Puccinia* spp.)

In this study, we demonstrated that TaEDR1 plays a negative role in plant immunity. Our work is a successful example of using genome editing to generate an agriculturally important trait in wheat. We are currently assessing the performance of *Taedr1* plants in the field, and challenging the resistance of these plants to various pathogens to better understand the breadth of their utility. The *Taedr1* plants that were generated will be valuable resources for future wheat breeding programs.

EXPERIMENTAL PROCEDURES

Plant materials and powdery mildew isolates

Bread wheat KN199, a semi-winter cultivar (http://www.most.gov.cn/kjbgz/201102/t20110215_84793.htm), was used for gene cloning, as a control in powdery mildew infections and for functional gene analysis. Diploid wheat *Triticum urartu* (G1812 and PI428309) (Ling *et al.*, 2013), *Aegilops speltoides* (Y606) and *Aegilops tauschii* (Y2281) (Jia *et al.*, 2013) were also used to clone *EDR1*, which was used for phylogenetic analysis. All plant materials were grown in a greenhouse under a 16-h/8-h light cycle at a constant 20°C.

The *Bgt* isolate E09 (Zhou *et al.*, 2005) was obtained from Xiangqi Zhang, Chinese Academy of Sciences and maintained on KN199 in a growth chamber with a 16-h/8-h light cycle and 70% humidity, at a constant 20°C.

RNA extraction and gene expression analysis

Total RNA was extracted from the leaves of KN199 using TRIZOL (Invitrogen, http://www.invitrogen.com/). To clone *TaEDR1* homoeologs, high-quality first-strand cDNA was generated according to the SMARTer RACE 5′/3′ Kit User Manual (Clontech, http://www.clontech.com/). For gene expression analysis, quantitative reverse transcription PCR (qRT-PCR) was performed on a Mastercycler ep realplex^2 (Eppendorf, https://www.eppendorf.com/). Specific primers for each gene were designed using PRIMER 3.0, and synthesized by Sangon Biotech (https://www.sangon.com/) (Table S2). Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, http://www.promega.com/) was used for reverse transcription and SYBR Premix Ex Taq (TaKaRa, http://www.takara-bio.com/) was used for qRT-PCR assays. The expression levels of target genes were normalized to *TaACTIN*. The statistical significance was evaluated by Student's *t*-test ($P < 0.05$).

Cloning of three *TaEDR1* homoeologs and sequence analysis

The homoeologs of *TaEDR1* were cloned by a homology cloning strategy. Specific primers from ATG to TGA were designed according to the open reading frames of *T. aestivum EDR1* (*TaEDR1*) mRNA (AY743662.2) (Niu *et al.*, 2005), and the KAPA HiFi DNA Polymerase (Kapa Biosystems, https://www.kapabiosystems.com/) was used to amplify all alleles of *TaEDR1*. The PCR products were subcloned into pEASY-BLUNT vector (TransGen Biotech, http://www.transgenbiotech.com/) and sequenced (Invitrogen). The sequencing results were aligned with the genome of the bread wheat cultivar 'Chinese Spring' using the service provided by the International Wheat Genome Sequencing Consortium (https://urgi.versailles.inra.fr/blast/blast.php), and the chromosomal locations were predicted.

BSMV-VIGS treatment

A BSMV-VIGS system was adopted to transiently silence endogenous *TaEDR1* (Scofield *et al.*, 2005). A $\gamma_{gammab:TaEDR1as}$ construct (Figure S6), harboring a 236-bp antisense fragment of *TaEDR1* was created using $\gamma_{gammab:GFP}$ cDNA as the starting material, as described previously (Zhou *et al.*, 2007). The $\gamma_{gammab:GFP}$ vector was used as a control. The viral RNA molecules were transcribed *in vitro* from the α, β and γ linearized plasmids using a RiboMAX Large Scale RNA Production-T7 kit (Promega, http://www.promega.com/) with the addition of a cap analog (Promega). The inoculation was performed on the fully expanded second leaf of two-leaf stage wheat plants with a 1:1:1 mixture of RNA_{α}, RNA_{β} and $RNA_{\gamma:TaEDR1}$ ($\gamma_{gammab:TaEDR1as}$) or $RNA_{\gamma:GFP}$ ($\gamma_{gammab:GFP}$) by mechanical rubbing. The inoculated plants were grown under standard conditions for about 20 days, at which point the fourth leaves were collected. We used qRT-PCR to confirm the effectiveness of the VIGS approach (Holzberg *et al.*, 2002; Scofield *et al.*, 2005) .

Generation and characterization of *TaEDR1* RNAi wheat plants

The RNAi construct for transgenic plants is based on the pAHC25 vector (Cao *et al.*, 2011). Both sides of the sense and antisense 196-bp *TaEDR1* sequences were flanked by a *TaWaxy* intron as a linker (Li *et al.*, 2005), and pBluscript II SK (−) was used as an intermediate donor. Firstly, the sense and antisense 196-bp fragments of *TaEDR1* were amplified with primers with added restriction sites (*TaEDR1*-TRi-S with 5′-*Sma*I and *Sac*I, *TaEDR1*-TRi-A

with 5′-*Bam*HI and *Spe*I; Table S2). A 151-bp segment of the *TaWaxy* gene was amplified as an intron (primers: *TaWaxy*-intron-S and *TaWaxy*-intron-A; Table S2) and introduced into the intermediate vector pBluscript II SK (−) using restriction sites *Bam*HI and *Spe*I. This vector was designated as pBS-*Wx*. Then, the sense fragment of *TaEDR1* and pBS-*Wx* were ligated in *Sac*I and *Spe*I sites, and designated as pBS-sense-*Wx*. *TaEDR1* antisense fragment and pBS-sense-*Wx* were cleaved by *Bam*HI and *Sma*I, and their ligated products were named pBS-sense-*Wx*-antisense. As a donor, pBS-sense-*Wx*-antisense was digested with *Sma*I and *Sac*I, and the sense-*Wx*–antisense fragment was ligated with the pAHC25 vector in the same sites (Figure S7) (Cao *et al.*, 2011). The transcribed RNA contains a hairpin sequence that forms dsRNA.

For silencing, the pAHC25-sense-*Wx*-antisense construct, which also contained the *BAR* (phosphinothricin acetyl transferase from *Streptomyces hygroscopicus*) gene was bombarded into calli cultured from immature embryos of the *Bgt*-susceptible variety KN199 using a PDS1000/He particle inflow gun (Bio-Rad, http://www.bio-rad.com/), as described previously (Brunner *et al.*, 2011; Liu *et al.*, 2013). After 2 weeks of dark cultivation, T_0 generation plants were screened on medium containing phosphinothricin (PPT). Herbicide-resistant plants were cultivated and analyzed for the presence of the *BAR* gene. T_1 plants were screened by *BAR* gene amplification, and *TaEDR1* transcript levels were detected by qRT-PCR. RNA was collected from at least three biological replicates. Primers were designed with PRIMER 3.0 (Table S2).

Pathogen inoculation and evaluation for plant resistance

Powdery mildew infection and microscopy analyses were performed as previously reported (Wang *et al.*, 2014) with minor modifications. Leaf segments of length 5 cm were detached from the main stem and immediately placed into Petri dishes containing 1% (w/v) distilled water agar and 50 mg L^{-1} benzimidazole. After 4 h the leaf segments were sprayed with *Bgt* E09 spores. Seventy-two hours post-inoculation, the inoculated leaves were stained with trypan blue dye in boiling water for 10 min and cleared with deionized water. The MI was determined using an Olympus BX52 microscope. Both the successfully colonized *Bgt* spores and spores that did not form colonies were counted. The *Bgt* MI represents the percentage of successfully colonized *Bgt* out of all analyzed spores (Liu *et al.*, 2016).

Forty-eight hours post-inoculation, the accumulation of H_2O_2 in plant cells was detected by staining with DAB (Sigma-Aldrich, http://www.sigmaaldrich.com/) as described previously (Xing *et al.*, 2013). Leaves were soaked in 1 mg ml^{-1} DAB solution (pH 3.8) for 16 h at 25°C in the dark, and de-colored in boiling 95% ethanol for 10 min. The cleared samples were characterized under an Olympus BX52 microscope. Callose deposition in the infected leaves was detected using aniline blue diammonium salt (AB; Sigma-Aldrich). Before microscopic examination, leaves at 48 h post-inoculation were first de-colored using 95% ethanol and then stained in 0.01% aniline blue solution dissolved in 150 mM K_2HPO_4 (pH 9.5) for 8 h (Xing *et al.*, 2013; Wu *et al.*, 2015). Trypan blue staining was also used to observe the mycelium structures at 7 dpi.

Selection of single-guide RNA (sgRNA) targets

Several sgRNA targets for *TaEDR1* were designed on the conserved domains of all three genomes of bread wheat. The activities of the sgRNAs were evaluated by co-transforming the pJIT163-Ubi-Cas9 (Wang *et al.*, 2014) and TaU6-sgRNA (Shan *et al.*, 2013b) plasmids into wheat protoplasts. Wheat protoplasts were isolated and transformed as previously described (Shan *et al.*, 2014). Protoplast transformation was carried out with a mixture of 10 μg pJIT163-Ubi-Cas9 and 10 μg TaU6-sgRNA plasmid.

Generation and identification of CRISPR/Cas9 mutants

Biolistic transformation of plasmids (mixed in a 1:1 molar ratio for Cas9 and sgRNA) into wheat immature embryos was performed using a PDS1000/He particle bombardment system (Bio-Rad) (Rasco-Gaunt *et al.*, 2001). After bombardment, embryos were cultivated and identified using a pooling method as previously described (Zhang *et al.*, 2016). No selective agents were used in any part of the tissue culture process.

ACCESSION NUMBERS

Sequence data from this article can be found in GenBank databases under the following accession numbers: AAU89661.2 (TaEDR1-1A), AAG31142.1 (HvEDR1), XP_003573947.1 (BdEDR1), XP_004983057.1 (SiEDR1), AAN61142.1 (OsEDR1), XP_008651221.1 (ZmEDR1), ABR45974.1 (AtEDR1).

ACKNOWLEDGEMENTS

We thank Xiangqi Zhang for BSMV constructs and *Bgt* isolate E09. The work was supported by grants from National Science Fund for Distinguished Young Scholars of China (31525019), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB11020100), the Ministry of Science and Technology of China (2015CB910202 and 2014DFA31540) to DT, the National Key Research and Development Program of China (2016YFD0101804) and the Chinese Academy of Sciences (KFZD-SW-107) to CG.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The three homoeologs of *TaEDR1* are located on chromosomes 1A, 1B and 1D.

Figure S2. Sequence alignments of TaEDR1 homoeologs in *Blumeria graminis* f. sp. *tritici* isolate E09 susceptible (KN199, Chinese Spring, Xiaoyan 54) and resistant (Xiaobaidong Mai, Youbailan, Fuzhuang) varieties of hexaploid wheat.

Figure S3. Alignment of TaEDR1 and EDR1 from *Triticum urartu*, *Aegilops speltoides tausch* and *Aegilops tauschii*.

Figure S4. Phylogenetic tree of EDR1 proteins.

Figure S5. PCR-RE results for seven potential *TaEDR1* CRISPR/Cas9 off-target sites in six T_1 mutants.

Figure S6. The BSMV α, β, $\gamma_{gammab:GFP}$, and $\gamma_{gammab:TaEDR1as}$ vectors used in this study. The *TaEDR1* target sequence is listed in the square and was inserted into the γ vector with *Nhe*I sites (underlined sequence).

Figure S7. The *TaEDR1* RNAi vector, pAHC25-sense-*Wx*-antisense, used in this study.

Figure S8. KN199 and triple *EDR1* knockout plant were indistinguishable at tillering and jointing growth stages in the field.

Table S1. CRISPR/Cas9-induced mutations in *TaEDR1* (left) and the transmission rate to the T_1 generation (right).

Table S2. Primers used in this study.

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