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Brief Communication

Engineering null mutants in *ZmFER1* confers resistance to ear rot caused by *Fusarium verticillioides* in maize

Changlin Liu[1,2,*,†], Ming Kong[1,†], Jinjie Zhu[1], Xiantao Qi[1], Canxing Duan[1] and Chuanxiao Xie[1,2,*]

[1]*Institute of Crop Science, Chinese Academy of Agricultural Sciences, National Key Facility for Crop Gene Resources and Genetic Improvement, Beijing, China*
[2]*Hainan Yazhou Bay Seed Lab, Sanya, China*

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**Correspondence* (Tel +86 10 82107464; fax +86 10 82106748; email xiechuanxiao@caas.cn (C.X.); Tel +86 10 82106748; fax +86 10 82106748; email liuchanglin@caas.cn (C.L.))
†These authors contributed equally to this work.

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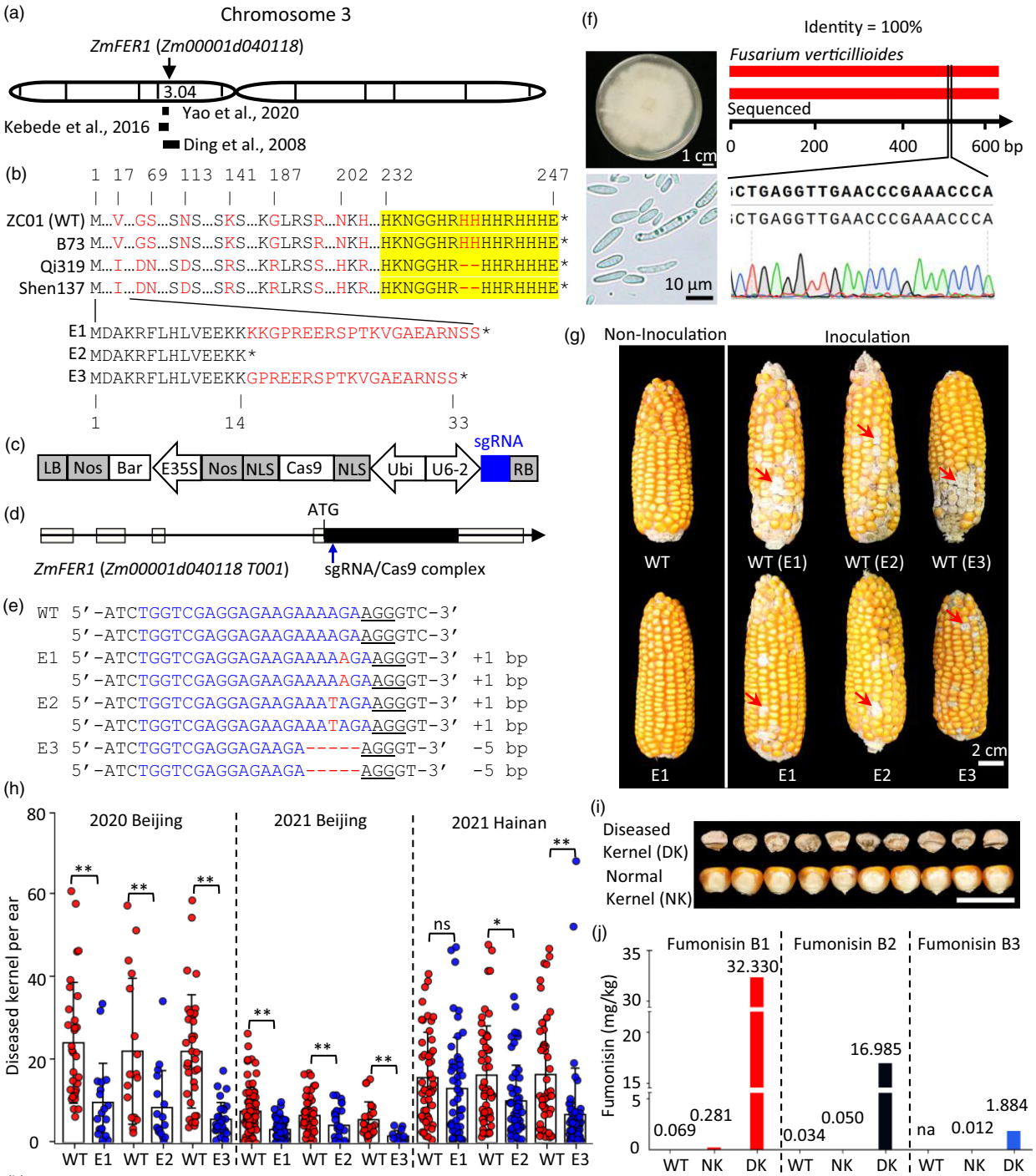
Fusarium ear rot (FER), a fungal disease caused by *Fusarium* spp., leads to great yield losses to maize worldwide and to mycotoxin contamination, which seriously threatens human and animal health (Yao *et al*., 2020). FER has become an especially serious problem in maize–wheat rotation systems because the major causal agent can also lead to *Fusarium* head blight (FHB) in wheat (Li *et al*., 2019). Employing resistant cultivars is the most effective method for controlling FER but is currently limited to only a few resistance genes. *Fhb1*, a major quantitative trait locus (QTL) for FHB resistance, was cloned recently (Li *et al*., 2019; Su *et al*., 2019). Although the reported resistance mechanisms were controversial, *TaHRC* underlying *Fhb1* was confirmed to be a susceptibility gene; a 752-bp deletion in this gene led to a loss of susceptibility (Li *et al*., 2019; Su *et al*., 2019). Knockout of the susceptible *TaHRC* allele improved FHB resistance (Chen *et al*., 2022; Su *et al*., 2019). Because FER and FHB could be caused by the same fungus (Li *et al*., 2019; Yao *et al*., 2020), we hypothesized that engineering *TaHRC* homologues in maize could represent a novel approach to FER resistance breeding.

Two *TaHRC* homologues have been identified in maize: *Zm00001d040118* (designated as *ZmFER1*) and *Zm00001d008500* (Li *et al*., 2019). Several QTLs underlying resistance to FER have also been identified to be adjacent to or overlap with *ZmFER1* (Ding *et al*., 2008; Kebede *et al*., 2016; Yao *et al*., 2020; Figure 1a). Using the resistant maize inbred lines Shen137 and Qi319 (Hu *et al*., 2021) and the susceptible line B73 (Kebede *et al*., 2016) and wild-type ZC01 (WT), we identified 12 polymorphisms in the coding sequence of *ZmFER1*, resulting in two haplotypes (Figure 1b). A 6-bp deletion led to the absence of two amino acids in the *ZmFER1* histidine-rich region in the resistant lines (Figure 1b). By contrast, no polymorphism was detected in the coding sequence of *Zm00001d008500* among the four inbred lines (data not shown). Based on these results, we reasoned that *ZmFER1* might participate in FER resistance in maize.

To test this hypothesis, we constructed a CRISPR/Cas9 vector (Figure 1c) targeting *ZmFER1* ~25 bp downstream of the translation start codon (Figure 1d) and stably transformed WT with this CRISPR/Cas9 vector. Using targeted PCR and sequencing, we selected three T_0 homozygous events: E1 (1-bp insertion), E2 (1-bp insertion) and E3 (5-bp deletion), which were predicted to produce truncated proteins of 35, 14 and 33 amino acids, respectively (Figure 1b,e). We selected transgene-free individuals from T_1 plants lacking Cas9 and Bar, as described previously (Liu *et al*., 2022) and used the T_1 and transgene-free T_2 and T_3 plants for pathogen resistance assays.

Fusarium verticillioides was used as the pathogen (Figure 1f). The sequence of a 654-bp fragment on chromosome 1 in the gene (GeneID: 30067583) of the pathogen was 100% identical to the *Fusarium verticillioides* sequence at NCBI (accession no. NC_031675.1) (Figure 1f). We mixed seeds from the WT and each mutant separately at a 1:1 ratio for blind artificial inoculation. Eight days after self-pollination, we performed inoculation of the ears through the silk channel using a 2 mL conidial suspension (1×10^6 spores/mL) and covered each ear with a bag to reduce natural infection (Ding *et al*., 2008). We evaluated FER resistance by counting diseased kernels per ear (DKPE) at 40 days after inoculation. We performed artificial inoculation in three environments: Beijing (39.9°N, 116.3°E) in the summer of 2020, Beijing in the summer of 2021, and Hainan (18.1°N, 109.3°E) in the winter of 2021. The non-inoculated WT and E1 ears showed no symptoms of FER (Figure 1g). By contrast, inoculated WT, E1, E2 and E3 ears showed obvious symptoms of FER, including 'starburst' kernels, and scattered or groups of kernels with white mould (Figure 1g). These results indicated the effectiveness of artificial inoculation.

To evaluate the resistance of mutants to FER, we sequenced the target site of *ZmFER1* in all inoculated ears and counted the DKPE values. The ears were categorized into two groups (WT and mutant) based on the genotype of *ZmFER1*. We determined the difference in DKPE between the two groups using Student's *t*-test. In Beijing in the summer of 2020, the differences in DKPE between the WT and mutants were all statistically significant at $P = 0.01$ (Figure 1h), with average DKPE values of 28.65 and 9.54 for the WT and mutants, respectively. In Beijing in the summer of 2021, the DKPE values for E1, E2 and E3 were also significantly different from the WT at $P = 0.01$ (Figure 1h), with average DKPE values of 8.70 and 4.08 for the WT and mutants, respectively. In Hainan in the winter of 2021, the DKPE values for E2 and E3 were also significantly different from the WT, but the DKPE for E1 was not. This is consistent with the finding that FER resistance is significantly influenced by the environment (Yao *et al*., 2020). As determined using the National Standards of the People's Republic of China (GB5009.240-2016), diseased kernels had much higher fumonisin contamination than normal kernels (Figure 1i,j), indicating the serious infection of *Fusarium verticillioides*. We also investigated the agronomic traits of the lines without inoculation in the field using a random-block design



(k)

Line	Plant height (n=40, cm)	Ear height (n=40, cm)	Leaf number (n=40)		Hundred kernel weight (n=40, g)	Kernel size (n=100, mm)		
			Below ear	Above ear		Length	Width	Thickness
WT	200.75±6.24 ^{a}	92.80±8.45 ^{a}	7.25±0.67 ^{b}	4.93±0.34 ^{c}	26.02±3.07 ^{a}	11.02±0.53 ^{a}	7.86±0.63 ^{b}	4.05±0.50 ^{c}
E1	199.38±7.92 ^{a}	91.28±6.49 ^{a}	7.38±0.59 ^{a}	5.20±0.41 ^{b}	26.56±3.01 ^{a}	11.16±0.54 ^{a}	8.14±0.60 ^{a}	4.21±0.50 ^{c}
E2	193.65±5.31 ^{b}	87.55±5.62 ^{b}	7.35±0.53 ^{a}	5.1±0.38b ^{c}	25.98±4.96 ^{a}	10.52±0.61 ^{c}	8.05±0.68 ^{a}	4.44±0.72 ^{b}
E3	193.98±7.47 ^{b}	82.33±8.86 ^{c}	6.90±0.55 ^{c}	5.23±0.51 ^{a}	26.50±1.39 ^{a}	10.68±0.52 ^{b}	7.99±0.53 ^{ab}	4.81±0.73 ^{a}

with two repeats. Although some traits of the mutants were different from that of the WT, no obvious agronomic penalty was observed (Figure 1k).

In summary, we demonstrated that the targeted generation of null mutants in *ZmFER1* could improve resistance against *Fusarium verticillioides* without agronomic penalty. These results

Figure 1 Targeted editing of *ZmFER1* confers resistance against *Fusarium verticillioides* in maize. (a) QTLs adjacent to or overlapped *ZmFER1*. (b) Sequence comparison of *ZmFER1* in the susceptible line B73, the resistant lines Qi319 and Shen137 and mutants. The histidine-rich region is highlighted in yellow. (c) Vector used in this study, described by Liu *et al*. (2022). (d) Structure and target site of *ZmFER1*. (e) Genotypes of the selected homozygous mutants. (f) *Fusarium verticillioides* culture used in the present study. (g) Comparison of non-inoculated and inoculated ears. Red arrows, diseased kernels. WT (E1), WT (E2) and WT (E3), the corresponding WT of E1, E2 and E3, respectively. (h) Comparison of diseased kernels per ear. *, $P < 0.05$. **, $P < 0.01$. (i) Comparison of diseased versus normal kernels. Bar, 2 cm. (j) Fumonisin levels in WT kernels without inoculation, normal kernels (NK) and diseased kernels (DK) separated from inoculated ears. (k) The mutants show no agronomic penalty. The differences in agronomic traits among WT, E1, E2 and E3 were compared using Duncan's multiple range test at $P = 0.05$. The superscript letters of a, b, and c indicate stastistically significant differences.

provide a new approach for breeding FER resistant cultivars, which could have huge benefits, especially in intensive maize–wheat rotation systems.

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Conflict of interest

A related patent had been submitted to the State Intellectual Property Office of China.

Author contributions

CL, MK, JZ, XQ, CD and CX performed the experiments. CX and CL wrote the manuscript.

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