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Brief Communication

Targeted generation of Null Mutants in *ZmGDIα* confers resistance against maize rough dwarf disease without agronomic penalty

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Introduction

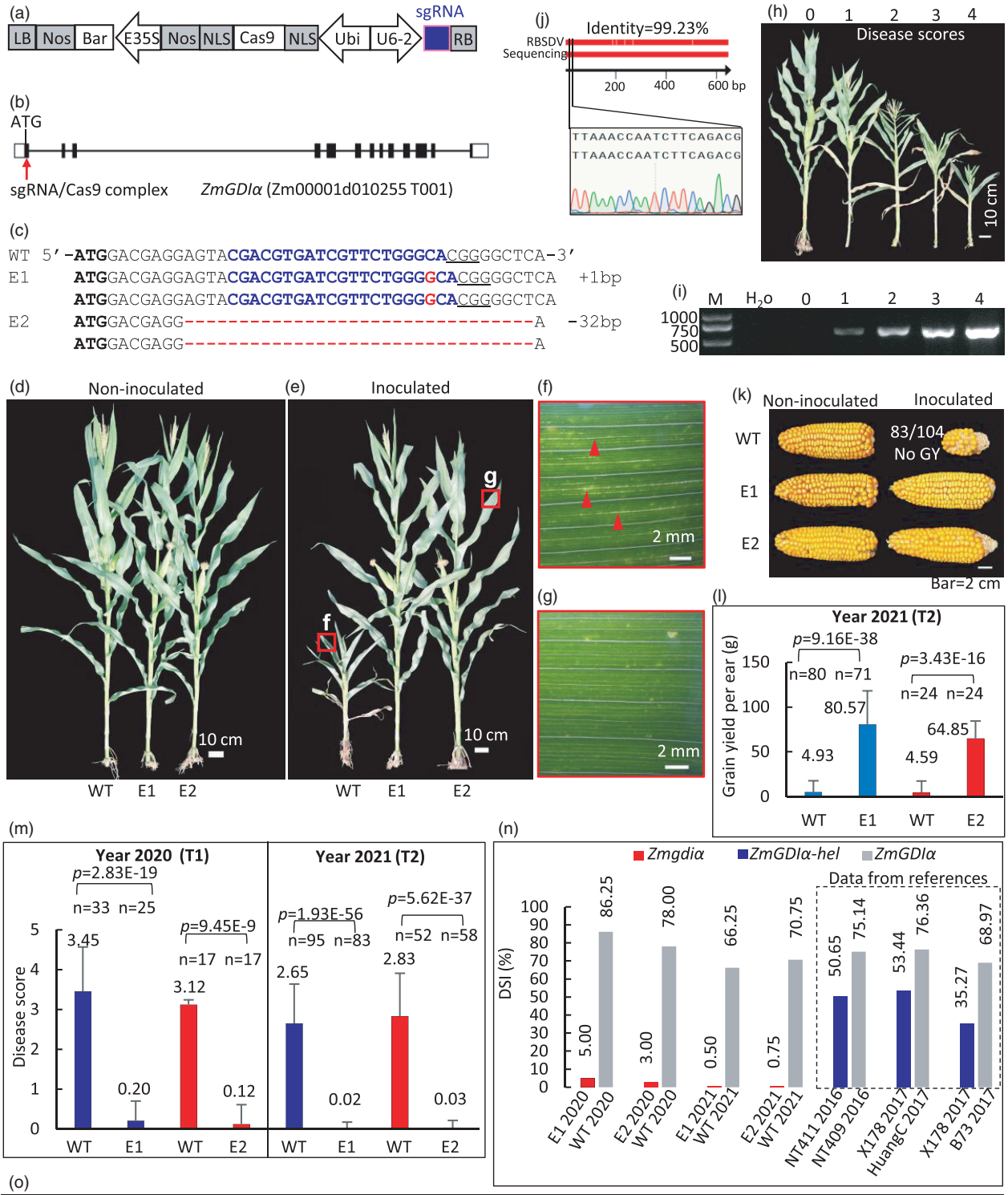
Maize rough dwarf disease (MRDD) is a worldwide disease caused by a virus (Bai *et al*., 2002; Harpaz, 1959). Rice black-streaked dwarf virus (RBSDV) was identified as the major pathogen causing MRDD in maize (*Zea mays*) and dwarfing disease in other cereal crops, seriously threatening crop production in Asia (Bai *et al*., 2002). The recessive allele conferring MRDD resistance has been cloned and characterized (Liu *et al*., 2020). The natural variant with an alternative exon 10 caused by a helitron transposon insertion, designated *ZmGDIα-hel*, weakened the interaction between the RBSDV P7-1 protein and the encoded ZmGDIα-hel protein, leading to quantitative resistance in maize plants. True loss-of-function alleles in *ZmGDIα* were expected to confer MRDD resistance at the cost of deleterious effects (Liu *et al*., 2020), as GDIα regulates small Rab GTPases, which are critical for vesicle membrane trafficking in eukaryotes (Schalk *et al*., 1996). However, plant Rab GTPases form the largest protein family and have evolved a unique set of 8 RAB sub-families with divergent profiles in monocot and dicot species (Tripathy *et al*., 2021). Essential but redundant plant factors, such as Eukaryotic Translation Initiation Factor 4E, have been a major target for engineering viral resistance without affecting plant fitness (Bastet *et al*., 2017). We therefore aimed to explore the possibility of generating null alleles of *ZmGDIα* to engineer viral resistance. Our efforts may provide a novel approach to MRDD resistance breeding beyond the natural allele of *ZmGDIα-hel*.

To generate a null mutant line, we constructed a CRISPR/Cas9 vector (Figure 1a) targeting exon 1 of *ZmGDIα* (Figure 1b) about 30 bp downstream of the translation start codon (Figure 1c). Stable transformation of maize inbred line ZC01 was performed as previously described (Li *et al*., 2017). We obtained 61 independent T_0 maize plants that contained both the *Bar* and *Cas9* genes, as determined by PCR amplification. Of those, 25 T_0 plants harboured mutations at the intended target site based on PCR and sequencing. We selected the two edited event T0 plants E1 and E2, which were homozygous for a 1-bp insertion (E1) or a 32-bp deletion (E2), respectively (Figure 1c). The T_1 plants were screened for transgene-free with neither *Cas9* nor *Bar*. The target sequencing data of both *zmgdiα* mutants confirmed their stable inheritance across the T_0, T_1 and T_2 generations. The T_1 and transgene-free T_2 mutant plants were characterized further.

We inoculated maize seedlings with RBSDV and then transplanted them to the field (Liu *et al*., 2016). The non-inoculated edited plants showed no obvious differences compared to WT plants (Figure 1d). In sharp contrast, inoculated WT plants had much shorter overall height and internodes compared to E1 and E2 plants (Figure 1e). In addition, we only observed another typical MRDD symptom consisting of waxy enations on the abaxial surface of WT upper leaves (Figure 1f) but not in the mutants (Figure 1g), thus validating their resistant phenotype.

We mixed seeds from the WT and each null mutant line separately in a 1:1 ratio for blind artificial inoculation, transplanting and field phenotyping to exclude possible bias. We assigned a five-grade disease score (Liu *et al*., 2016) to each plant (Figure 1h). We ascertained that the virus titre is proportional to MRDD severity (Figure 1i) by RT-PCR using the primer pair that is specific to a 652-bp region in the S4 segment of RBSDV. Sanger sequencing of the amplicon yielded the sequences that were 99.2% identical to the deposited RBSDV sequence at NCBI (#KY662121.1), confirming infection by this virus (Figure 1j).

We sequenced *ZmGDIα* in all blind-mixed individuals for genotyping and scoring agronomic traits. We then calculated and compared the agronomic performance (Figure 1k, l) and disease score (Figure 1m) of the WT and mutant groups. Most inoculated WT plants produced few to no kernels. By contrast, inoculated edited mutant lines bore ears comparable to those seen on non-inoculated plants (Figure 1k). In 2020, we identified each WT:mutant ratios were a 1:1 ratio from blind-mixed population, as determined by a *chi*-square test at $P = 0.05$ (Figure 1m, left). The average disease score of the E1 line was significantly different from that of the corresponding WT plants, as was the average disease score of the E2 line relative to its corresponding WT. In 2021, we further repeated this analysis using about three times as many plants as in 2020. Again, we obtained WT:mutant ratios consistent with a 1:1 ratio. The average disease scores for E1 and E2 were significantly different from those of their corresponding WT, respectively (Figure 1m, right). The resistance conferred by *zmgdiα* was higher than that of previously reported resistant materials carrying *ZmGDIα-hel*, as



(o)

Line	Plant height (cm)	Ear height (cm)	Leaf number		HKW (g)	Kernel size (mm)		
			Below ear	Above ear		Length	Width	Thickness
WT (n=23)	222.39±8.39 [a]	117.09±6.56 [a]	7.9±0.46 [a]	5.4±0.50 [a]	24.90±0.15 [a]	10.53±0.59 [a]	8.01±0.48 [a]	4.65±0.38 [a]
E1 (n=27)	223.67±8.08 [a]	113.67±6.75 [a]	8.2±0.46 [a]	5.2±0.48 [a]	24.87±0.13 [a]	10.28±0.29 [a]	8.11±0.27 [a]	4.67±0.43 [a]
E2 (n=19)	218.11±7.05 [a]	116.79±5.22 [a]	8.0±0.40 [a]	5.2±0.42 [a]	24.92±0.15 [a]	10.45±0.49 [a]	8.27±0.28 [a]	4.77±0.45 [a]

Note: [a], not statistically significant at P=0.05

Figure 1 Genome editing *ZmGDIα* confers resistance against MRDD without agronomic penalty. (a) Constructed vector. (b) The designed target site. (c) Genotype of the selected homozygous mutations. (d, e) Comparison of non-inoculated (d) and inoculated (e) plants. The leaves in the red rectangles in (e) are magnified to show MRDD symptoms in the WT (f) and E2 (g). (f, g) Typical waxy enation symptom of MRDD is seen on the WT (f) but not in mutant (g). (h) Representative plants with scored MRDD severity from 0 to 4 from resistance to susceptibility. (i) RT-PCR detection of RBSDV showing that the virus titre is proportional to scored MRDD severity. (j) Verification of RBSDV identity by sequencing RT-PCR amplicon in (i). (k, l) Comparison of representative maize ears (k) and grain yield (l) in the field. Eighty-three out of 104 inoculated WT plants produced no grain. (m) Disease scores across years 2020 and 2021. (n) Comparison of DSI observed in the null mutants and the previously reported *ZmGDI-hel*. NT411/NT409 and X178/Huang C data were reproduced from Liu C. et al. (2020). X178/B73 data were reproduced from Liu Q. *et al*. (2016). DSI (%) = ∑(disease score × number of plants with this disease score) × 100 / (maximum disease score × total number of plants). (o) The edited lines show no agronomic penalty without MRDD infection in the field. The differences were not statistically significant at $P = 0.05$.

evidenced by their much lower disease severity index (DSI) (Liu C. *et al*., 2016; Liu Q. *et al* 2020) (Figure 1n). These data indicated that the *ZmGDIα* locus identified by Liu *et al*. (2020) was a valuable target for engineering MRDD resistance and the generated null mutants might confer higher resistance.

To evaluate whether there are agronomic penalties due to the E1 or E2 mutations, we compared the agronomic performance under conditions free from RBSDV inoculation in the field, using a random-block design with two repeats each consisting of about 60 plants. We observed no obvious phenotypic differences for plant growth or development between the WT and the null *zmgdiα* mutants (Figure 1d). In addition, all other measured parameters were comparable between WT, E1, and E2 plants (Figure 1o).

In summary, our data indicate that both null mutants generated through CRISPR/Cas9 editing exhibit stronger resistance against MRDD than the natural *ZmGDIα-hel* allele. Our study also alleviates concerns about possible agronomic penalties associated with *ZmGDIα* loss-of-function mutants. Targeted editing of *Rab GDIα* might be extended in other monocot crops to engineer RBSDV resistance.

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Conflict of interest

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

Authors contribution

CL, MK, FY, JZ, XQ, JW, DD, and CX performed the experiments. CX and CL wrote the manuscript.

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