**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 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 T₀ maize plants that contained both the Bar and Cas9 genes, as determined by PCR amplification. Of those, 25 T₀ plants harbourcd 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 T₁ 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 T₀, T₁ and T₂ generations. The T₁ and transgene-free T₂ 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 WT plants produced few to no kernels. By contrast, inoculated edited plants bore ears comparable to those of their corresponding WT, respectively (Figure 1m, right). 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...
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(a) LB Nos Bar E35S Nos NLS Cas9 NLS Ubi U6-2 RB

(b) ATG

sgRNA/Cas9 complex ZmGD1a (Zm00001d010255 T001)

(c) WT 5′-ATGGACGAGGATACGCCGTTCTGGGCCAAGGGGCTCA-3′
E1 ATGGACGAGGATACGCCGTTCTGGGCCAAGGGGCTCA -3 bp
E2 ATGGACGAGGATACGCCGTTCTGGGCCAAGGGGCTCA -32 bp

(d) Non-inoculated
(e) inoculated

(f) Disease scores

(g) WT E1 E2

(h) Bar=2 cm

(i) M H2O 0 1 2 3

(j) Year 2021 (T2)

(k) Non-inoculated Inoculated

(l) Grain yield per ear (kg)

(m) Year 2020 (T1)

(n) Data from references...

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

Note: *, 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, IZ, XQ, JW, DD, and CX performed the experiments. CX and CL wrote the manuscript.

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