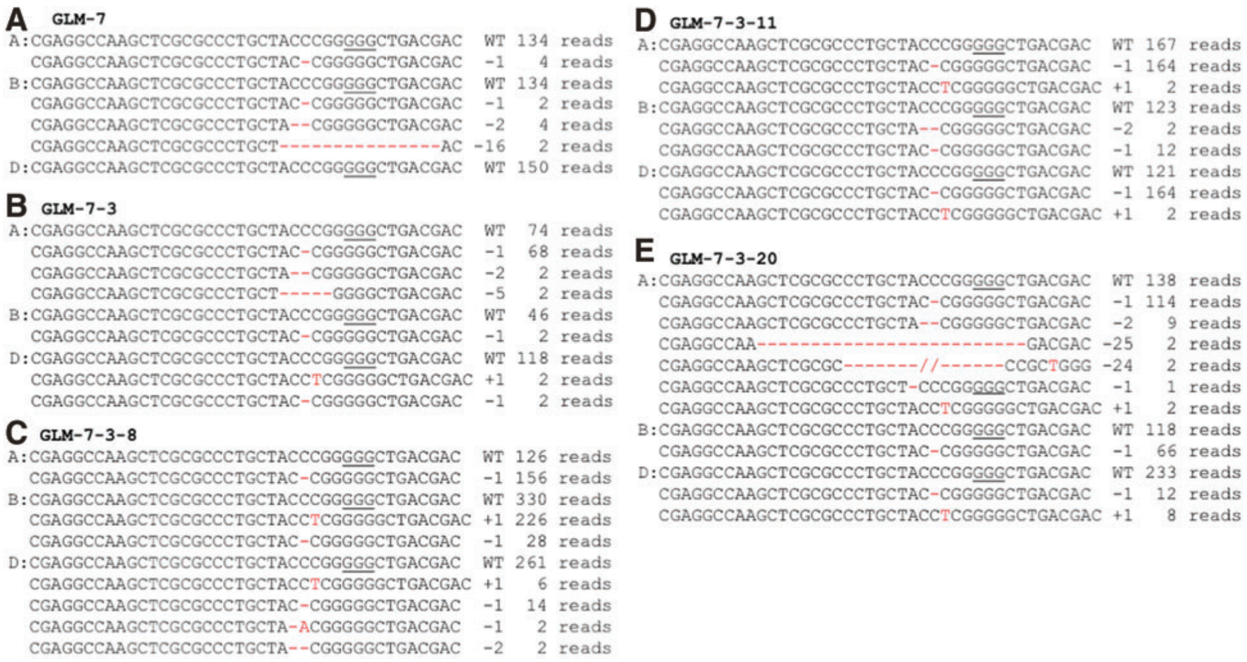


A GLM-7

```
A:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 134 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1   4 reads
B:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 134 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1   2 reads
  CGAGGCCAAGCTCGCGCCCTGCTA--CGGGGGCTGACGAC  -2   4 reads
  CGAGGCCAAGCTCGCGCCCTGCT---------------AC -16   2 reads
D:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 150 reads
```

B GLM-7-3

```
A:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT  74 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1  68 reads
  CGAGGCCAAGCTCGCGCCCTGCTA--CGGGGGCTGACGAC  -2   2 reads
  CGAGGCCAAGCTCGCGCCCTGCT-----GGGGCTGACGAC  -5   2 reads
B:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT  46 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1   2 reads
D:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 118 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1   2 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1   2 reads
```

C GLM-7-3-8

```
A:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 126 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1 156 reads
B:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 330 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1 226 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1  28 reads
D:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 261 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1   6 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1  14 reads
  CGAGGCCAAGCTCGCGCCCTGCTA-ACGGGGGCTGACGAC  -1   2 reads
  CGAGGCCAAGCTCGCGCCCTGCTA--CGGGGGCTGACGAC  -2   2 reads
```

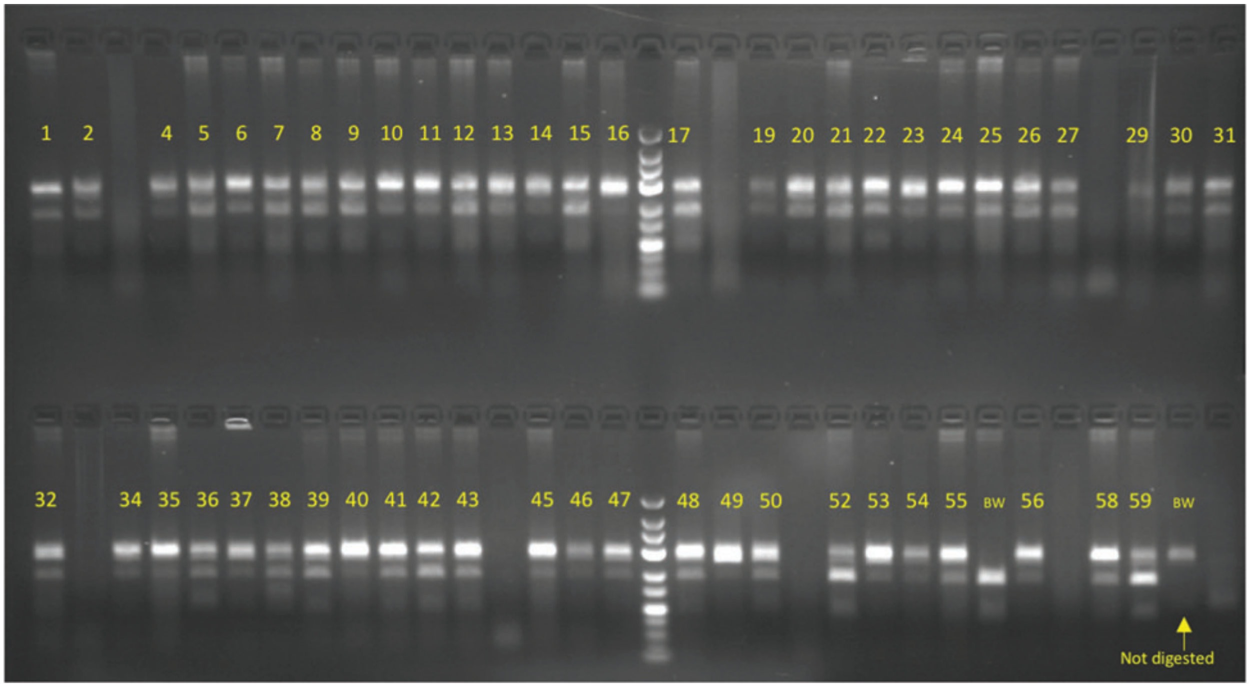
D GLM-7-3-11

```
A:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 167 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1 164 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1   2 reads
B:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 123 reads
  CGAGGCCAAGCTCGCGCCCTGCTA--CGGGGGCTGACGAC  -2   2 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1  12 reads
D:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 121 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1 164 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1   2 reads
```

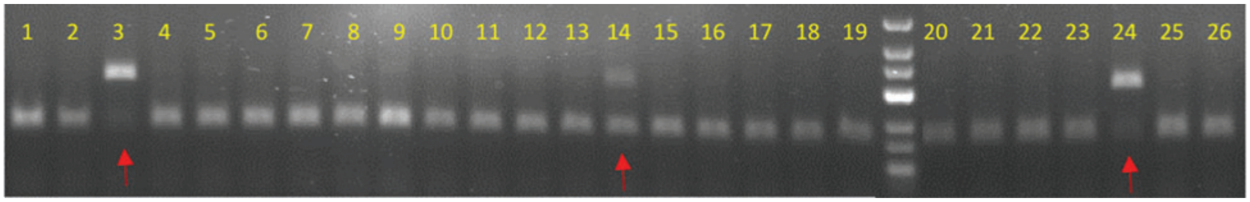
E GLM-7-3-20

```
A:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 138 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1 114 reads
  CGAGGCCAAGCTCGCGCCCTGCTA--CGGGGGCTGACGAC  -2   9 reads
  CGAGGCCAA-------------------------GACGAC -25   2 reads
  CGAGGCCAAGCTCGCGC-------//------CCGCTGGG -24   2 reads
  CGAGGCCAAGCTCGCGCCCTGCT-CCCGGGGGCTGACGAC  -1   1 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1   2 reads
B:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 118 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1  66 reads
D:CGAGGCCAAGCTCGCGCCCTGCTACCCGGGGGCTGACGAC  WT 233 reads
  CGAGGCCAAGCTCGCGCCCTGCTAC-CGGGGGCTGACGAC  -1  12 reads
  CGAGGCCAAGCTCGCGCCCTGCTACCTCGGGGGCTGACGAC +1   8 reads
```

SUPPLEMENTARY FIG. S7. Transgenerational CRISPR-Cas9 activity induces new mutations in the *TaGW2* gene. The GW2T2 target site was amplified and sequenced by NGS. All identified read types for **(A)** T_0 line GLM-7 and its **(B)** T_1, **(C–E)** T_2 progenies are shown. WT, wild-type alleles in wheat cultivar Bobwhite; "–" and "+" signs and numbers after them, nucleotides deleted and inserted, respectively. The detected numbers of each read type are shown on the right.



SUPPLEMENTARY FIG. S8. Restriction enzyme digestion of PCR amplicons to screen additional *gw2* knockout mutations in the T_3 progenies of line GLM-2-9-49. GW2T2 flanking region were amplified by PCR and digested with XmaI; non-digested PCR amplicons correspond to mutated GW2T2 target sites. The numbers on the gel image are identifiers of the GLM-2-9-49 progenies. Lane marked with arrows is PCR product from wild-type plant not digested with XmaI and loaded as control. BW, wild-type cultivar Bobwhite. From the top to the bottom, the DNA ladder fragment lengths are 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, 150 bp, and 100 bp.



SUPPLEMENTARY FIG. S9. Restriction enzyme digestion of PCR amplicons to screen mutations in the *TaLpx-1* gene in the T_2 progenies of line GLM-2-5. LPX1T2 flanking region was amplified by PCR and digested with SalI; non-digested PCR amplicons correspond to mutated LPX1T2 target sites. The numbers on the gel image are identifiers of the GLM-2-5 progenies. Lanes marked with arrows have non-digested PCR products. From the top to the bottom, the DNA ladder fragment lengths are 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, 150 bp, and 100 bp.