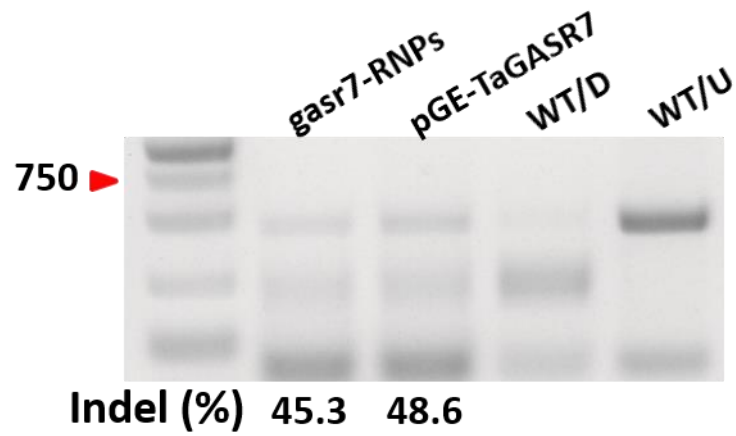


Supplementary Figure 1. *In vitro* cleavage of three homoeologs of *TaGW2* (*TaGW2-A1*, *-B1* and *-D1*) and *TaGASR7* (*TaGASR7-A1*, *-B1* and *-D1*) using gw2-RNPs or gasr7-RNPs. CK indicates the incubation without the addition of RNPs.



Supplementary Figure 2. Mutagenesis frequencies of *TaGASR7* (induced by *gasr7*-RNPs or pGE-*TaGASR7*) in wheat protoplasts analyzed by PCR-RE assay. WT/D and WT/U indicate wild type PCR amplicons with or without restriction enzyme digestion.

a

<i>TaGW2-A1</i> (pGE-TaGW2)		count	<i>TaGW2-A1</i> (gw2-RNPs)		count
WT:	CCTCTAGAAATGCCCCATCCTG		WT:	CCTCTAGAAATGCCCCATCCTG	
M1:	CCTCTAAGAAATGCCCCATCCTG	+1 996	M1:	CCTCTAAGAAATGCCCCATCCTG	+1 39
M2:	CCTCTATGAAATGCCCCATCCTG	+1 367	M2:	CCTCTATGAAATGCCCCATCCTG	+1 25
M3:	CCTCGA--AATGCCCCATCCTG	-2 330	M3:	CCTCT--GAAATGCCCCATCCTG	-1 23
M4:	CCTCTA----TGCCCCATCCTG	-4 284	M4:	CCTCT--AAATGCCCCATCCTG	-2 22
M5:	CCTCTA-----GCCCCATCCTG	-5 276	M6:	CCTC--GAAATGCCCCATCCTG	-2 5
M6:	CCTCTACGAAATGCCCCATCCTG	+1 141	M7:	CCTCTACGAAATGCCCCATCCTG	+1 4
M7:	CCTCT-GAAATGCCCCATCCTG	-1 129	M8:	CCTCT----ATGCCCCATCCTG	-4 4
M8:	CCTCT---AATGCCCCATCCTG	-3 120			
M9:	CCTCTA-AAATGCCCCATCCTG	-1 115			
M10:	CCTCTAGGAAATGCCCCATCCTG	+1 105			

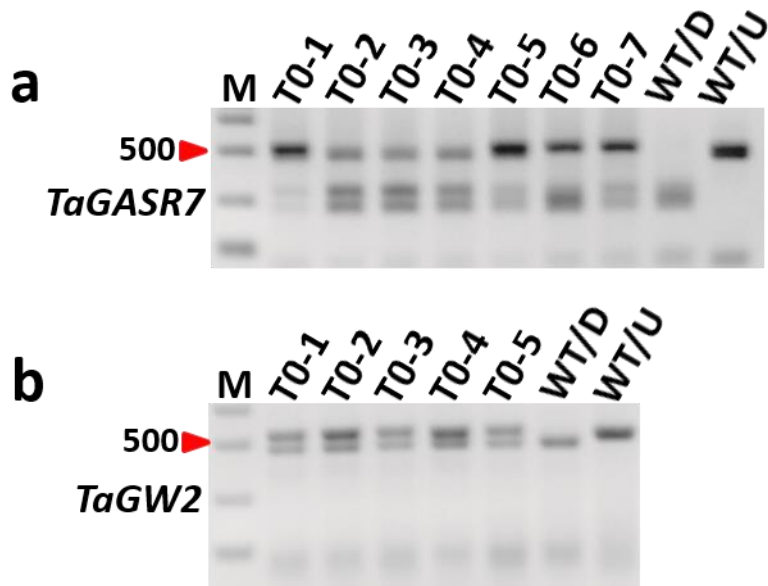
b

<i>TaGW2-B1</i> (pGE-TaGW2)		count	<i>TaGW2-B1</i> (gw2-RNPs)		count
WT:	CCTCTAGAAATACCCCATCCTG		WT:	CCTCTAGAAATACCCCATCCTG	
M1:	CCTCTAAGAAATACCCCATCCTG	+1 277	M1:	CCTCTAAGAAATACCCCATCCTG	+1 198
M2:	CCTCT---AATACCCCATCCTG	-3 200	M2:	CCTCTATGAAATACCCCATCCTG	+1 67
M3:	CCTCTATGAAATACCCCATCCTG	+1 184	M3:	CCTC--GAAATACCCCATCCTG	-2 43
M4:	CCTCT----ATACCCCATCCTG	-4 168	M4:	CCTCT--AAATACCCCATCCTG	-2 33
M5:	CCTCT--AAATACCCCATCCTG	-2 127	M5:	CCTCTACGAAATACCCCATCCTG	+1 28
M6:	CCTCTA-AAATACCCCATCCTG	-1 44	M6:	CCTCTA-AAATACCCCATCCTG	-1 21
M7:	CCTCTACGAAATACCCCATCCTG	+1 38	M7:	CCTCT--AAATACCCCATCCTG	-2 20
M8:	CCTCT-GAAATACCCCATCCTG	-1 37	M8:	CCTCT-GAAATACCCCATCCTG	-1 16
M9:	CCTCTA-----ACCCCATCCTG	-5 32	M9:	CCTCT---AATACCCCATCCTG	-3 12
M10:	C-----GAAATACCCCATCCTG	-5 28	M10:	CCTCTA---TTACCCCATCCTG	-4/+1 12

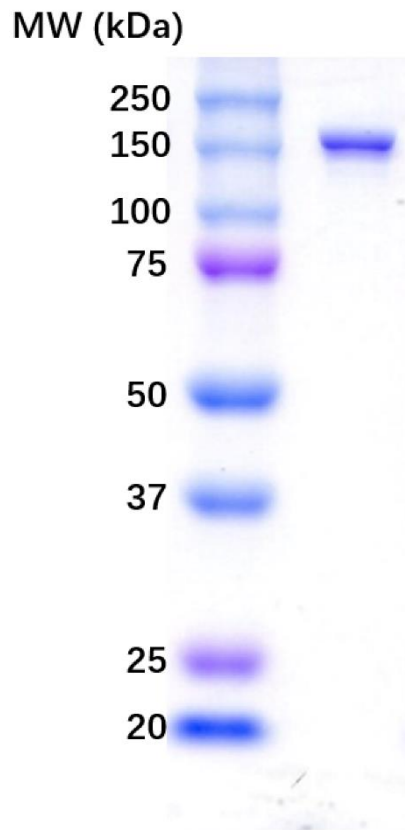
c

<i>TaGW2-D1</i> (pGE-TaGW2)		count	<i>TaGW2-D1</i> (gw2-RNPs)		count
WT:	CCTCTAGAAATACCCCATCCTG		WT:	CCTCTAGAAATACCCCATCCTG	
M1:	CCTCTAAGAAATACCCCATCCTG	+1 906	M1:	CCTCTAAGAAATACCCCATCCTG	+1 268
M2:	CCTCT---AATACCCCATCCTG	-3 652	M2:	CCTCTATGAAATACCCCATCCTG	+1 155
M3:	CCTCTATGAAATACCCCATCCTG	+1 515	M3:	CCTCT---AATACCCCATCCTG	-3 107
M4:	CCTCT----ATACCCCATCCTG	-4 500	M4:	CCTCT----ATACCCCATCCTG	-4 32
M5:	CCTCTACGAAATACCCCATCCTG	+1 212	M5:	CCTCTACGAAATACCCCATCCTG	+1 26
M6:	CCTCTA-----ACCCCATCCTG	-5 172	M6:	CCTCT--AAATACCCCATCCTG	-2 26
M7:	CCTCTAGGAAATACCCCATCCTG	+1 142	M7:	CCTCTACGAAATACCCCATCCTG	+1 23
M8:	CCTCTA-AAATACCCCATCCTG	-1 102	M8:	CCT---GAAATACCCCATCCTG	-3 12
M9:	CCTCTA----TGCCCCATCCTG	-4 64	M9:	CCTC--GAAATACCCCATCCTG	-2 8
M10:	CCTCTAG--ATACCCCATCCTG	-2 50	M10:	CCTCT-GAAATACCCCATCCTG	-1 7

Supplementary Figure 3. Ten most frequently occurred mutation types generated by pGE-TaGW2 or gw2-RNPs in immature embryos revealed by deep amplicon sequencing. Hyphens denote deleted nucleotides. The nucleotides inserted are labeled green. The PAM motif (CCT) is shown in red. The number on the right sides indicates the captured reads of the mutation type.



Supplementary Figure 4. PCR-RE assay analysis for *gasr7*-RNPs (a) and *gw2*-RNPs (b) induced mutants in hexaploid wheat line YZ814. Lanes T0-1 to T0-7 show the PCR products of the mutants after enzyme digestion. Lanes labeled by WT/D and WT/U are the PCR products amplified from wild type (WT) plants with and without enzyme digestion, respectively.



Supplementary Figure 5. SDS-PAGE image of purified Cas9 protein. 1 μ l Cas9 protein was detected by SDS-PAGE electrophoresis system using 10% separating gel.

Supplementary Table 1. sgRNA target loci and sequences.

Gene name	Target site (5'-3')	Oligo-F (5'-3')	Oligo-R (5'-3')	Detection method
<i>TaGW2</i>	<u>CCT</u> CTAGAAATACCCCATCCTG	CTTGCAGGATGGGGTA TTTCTAG	AAACCTAGAAATACCC CATCCTG	Deep sequencing and PCR-RE XbaI
<i>TaGASR7</i>	<u>CCG</u> CCGGGCACCTACGGCAAC	CTTGGTTGCCGTAGGT GCCCGG	AAACCCGGGCACCTAC GGCAAC	Deep sequencing and PCR-RE BclI

Supplementary Table 2. Mutagenesis frequencies of *TaGW2-A1*, *-B1*, *-D1* (induced by gw2-RNPs or pGE-TaGW2) and *TaGASR7* (induced by gasr7-RNPs or pGE-TaGASR7) in the embryos revealed by deep amplicon sequencing.

Target gene	Reagents	Total reads	Mutant reads	Mutagenesis frequency (%)
<i>TaGW2-A1</i>	pGE-TaGW2	425527	3245	0.76
	gw2-RNPs	390295	122	0.03
	Control	408883	10	0
<i>TaGW2-B1</i>	pGE-TaGW2	133533	1320	0.99
	gw2-RNPs	296538	541	0.18
	Control	220458	21	0.01
<i>TaGW2-D1</i>	pGE-TaGW2	433865	4333	1.00
	gw2-RNPs	323010	677	0.21
	Control	213139	19	0.01
<i>TaGASR7</i>	gasr7-RNPs	80240	448	0.56
	Control	17632	0	0

Supplementary Table 3. PCR primers used in this study.

Primer name	Primer sequence (5'-3')	Application
GW2-F GW2-R	ATGCCAACCCCTTGCGTGTGCGT TCCTGCTTGTGGGAGCTTTATG	Amplifying the <i>TaGW2</i> target site
GW2-A1-F	CTGCCATTACTTTGTATTTTGGTAATA	Amplifying the <i>TaGW2-A1</i> target site and 1 st PCR for deep sequencing
GW2-B1-F	G TTCAGATGGCAATCTAAAAGTT	Amplifying the <i>TaGW2-B1</i> target site and 1 st PCR for deep sequencing
GW2-D1-F	GCATGTACTTTGATTGTTTGCCTGA	Amplifying the <i>TaGW2-D1</i> target site and 1 st PCR for deep sequencing
GW2-A1/B1/D1-R	TCCTTCCTCTCTTACCACTTCCC	Amplifying the <i>TaGW2-A1</i> , <i>-B1</i> and <i>-D1</i> target site and 1 st PCR for deep sequencing
T7-GW2-F sgRNA-PCR-R	TAATACGACTCACTATAGGCAGGATGGGGTATT TCTAG GCACCGACTCGGTGCCACTT	Amplifying the T7-GW2-sgRNA
T7-GASR7-F sgRNA-PCR-R	TAATACGACTCACTATAGGGTTGCCGTAGGTGC CCGG GCACCGACTCGGTGCCACTT	Amplifying the T7-GASR7-sgRNA
GASR7-F GASR7-R	GGAGGTGATGGGAGGTGGGGG CTGGGAGGGCAATTCACATGCCA	Amplifying the <i>TaGASR7</i> target site and 1 st PCR for deep sequencing
GASR7-A1/B1/D1-F	CCTTCATCCTTCAGCCATGCAT	Amplifying the <i>TaGASR7-A1</i> , <i>-B1</i> and <i>-D1</i> target site
GASR7-A1-R	CCACTAAATGCCTATCACATACG	Amplifying the <i>TaGASR7-A1</i> target site
GASR7-B1-R	AGGGCAATTCACATGCCACTGAT	Amplifying the <i>TaGASR7-B1</i> target site
GASR7-D1-R	CCTCCATTTTTCCACATCTTAGTCC	Amplifying the <i>TaGASR7-D1</i> target site
BA1-GW2-AF BA2-GW2-AR	CGATGTTGCCTTTTGGCAACCAACG TGACCATCCATGCTTGATGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-A1</i> of pGE-TaGW2
BA3-GW2-BF BA4-GW2-BR	ACAGTGTGCCTTTTGGCAACCAACG GCCAATTCATGCTTGATGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-B1</i> of pGE-TaGW2
BA5-GW2-DF BA6-GW2-DR	CAGATCTGCCTTTTGGCAACCAACG CTTGATCCATGCTTGCTGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-D1</i> of pGE-TaGW2
BA13-GW2-AF BA14-GW2-AR	AGTCAATGCCTTTTGGCAACCAACG AGTTCCCTCCATGCTTGATGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-A1</i> of gw2-RNPs
BA15-GW2-BF BA16-GW2-BR	ATGTCATGCCTTTTGGCAACCAACG CCGTCCCTCCATGCTTGATGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-B1</i> of gw2-RNPs
BA17-GW2-DF BA18-GW2-DR	GTAGAGTGCCTTTTGGCAACCAACG GTCCGCTCCATGCTTGCTGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-D1</i> of gw2-RNPs
BA43-GW2-AF BA44-GW2-AR	TACAGCTGCCTTTTGGCAACCAACG TATAATTCCATGCTTGATGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-A1</i> of WT
BA45-GW2-BF BA46-GW2-BR	TCATTCTGCCTTTTGGCAACCAACG TCCCGATCCATGCTTGATGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-B1</i> of WT
BA47-GW2-DF BA48-GW2-DR	TCGAAGTGCCTTTTGGCAACCAACG TCGGCATCCATGCTTGCTGGCTGGTG	2 nd PCR for deep sequencing of <i>TaGW2-D1</i> of WT
BA41-GASR7-F BA42-GASR7-R	GCGCTAGAAGGCGTGCCTGACCTACT TAATCGCAAGAATCTAGGGGCACTTGG	2 nd PCR for deep sequencing of <i>TaGASR7</i> of WT
BA43-GASR7-F BA44-GASR7-R	TACAGCGAAGGCGTGCCTGACCTACT TATAATCAAGAATCTAGGGGCACTTGG	2 nd PCR for deep sequencing of <i>TaGASR7</i> of <i>gasr7</i> -RNPs

Supplementary Table 4. Genotypes of another 16 *tagw2* mutant plants induced by *gw2*-RNPs.

Plant ID	Genotype of <i>TaGW2-A1</i>	Genotype of <i>TaGW2-B1</i>	Genotype of <i>TaGW2-D1</i>
T0-13	AA	BB	Dd (-3 bp)
T0-14	AA	Bb (+1 bp)	dd (-9 bp)
T0-15	AA	Bb (+1 bp)	dd (-9 bp)
T0-16	AA	Bb (-1 bp)	Dd (+1 bp)
T0-17	AA	Bb (-1 bp)	Dd (+1 bp)
T0-18	AA	BB	Dd (-16 bp)
T0-19	AA	Bb (-6/+1 bp)	Dd (-6 bp)
T0-20	AA	Bb (-6/+1 bp)	Dd (-6 bp)
T0-21	AA	Bb (-6/+1 bp)	Dd (-6 bp)
T0-22	AA	BB	Dd (+1 bp)
T0-23	AA	BB	Dd (+1 bp)
T0-24	AA	BB	Dd (+1 bp)
T0-25	AA	BB	Dd (-3 bp)
T0-26	AA	BB	Dd (-3 bp)
T0-27	AA	BB	Dd (-6 bp)
T0-28	AA	BB	Dd (-6 bp)

Supplementary Table 5. Potential off-target sites analyzed for *TaGW2*.

Site name	Sequence	No. of mismatches	Mutagenesis frequency	Detection Method
On-target	<u>CCT</u> CTAGAAATACCCCATCCTG			
OT1	gAGGATGGGGTATTTaTAGTGG	2	0	Sanger Sequencing
OT2	tAaGATGGGtTATTTCTAGAGG	3	0	PCR-RE XbaI
OT3	gAGGATGGGaTtTTTCTAGAGG	3	0	PCR-RE XbaI
OT4	gAGGAgGGGGTATTTaTAGTGG	3	0	Sanger Sequencing
OT5	tAaGATGGGtTATTTCTAGAGG	3	0	Sanger Sequencing
OT6	CAGcATcGGGTATTTCTcGAGG	3	0	Sanger Sequencing
OT7	CtGGAcGGGGTATTTgTAGTGG	3	0	Sanger Sequencing
OT8	CAGGgTtGGGTcTTTCTAGTGG	3	0	Sanger Sequencing
OT9	gAGGcTGGGGTATTTaTAGTGG	3	0	Sanger Sequencing
OT10	CACgATGGcGcATTTCTAGTGG	3	0	Sanger Sequencing
OT11	aAGaATGGGccATTTCTAGAGG	4	0	PCR-RE XbaI
OT12	aAGaATGGGtcATTTCTAGAGG	4	0	PCR-RE XbaI
OT13	CgGcATGGaGTATaTCTAGAGG	4	0	PCR-RE XbaI
OT14	CcGGAgGGaGTATgTCTAGAGG	4	0	PCR-RE XbaI
OT15	ttGGAaGGGGgATTTCTAGAGG	4	0	PCR-RE XbaI
OT16	gAGGAcaGaGgATTTCTAGAGG	5	0	PCR-RE XbaI
OT17	CttGtgtGGGTATTTCTAGTGG	5	0	Sanger Sequencing
OT18	CAtGgTaGaGTtTTTCTAGTGG	5	0	Sanger Sequencing
OT18	tAaGATtGGGTATTgCTaTAGG	5	0	Sanger Sequencing
OT20	gAGcAgGcGGTATTTaTAGTGG	5	0	Sanger Sequencing