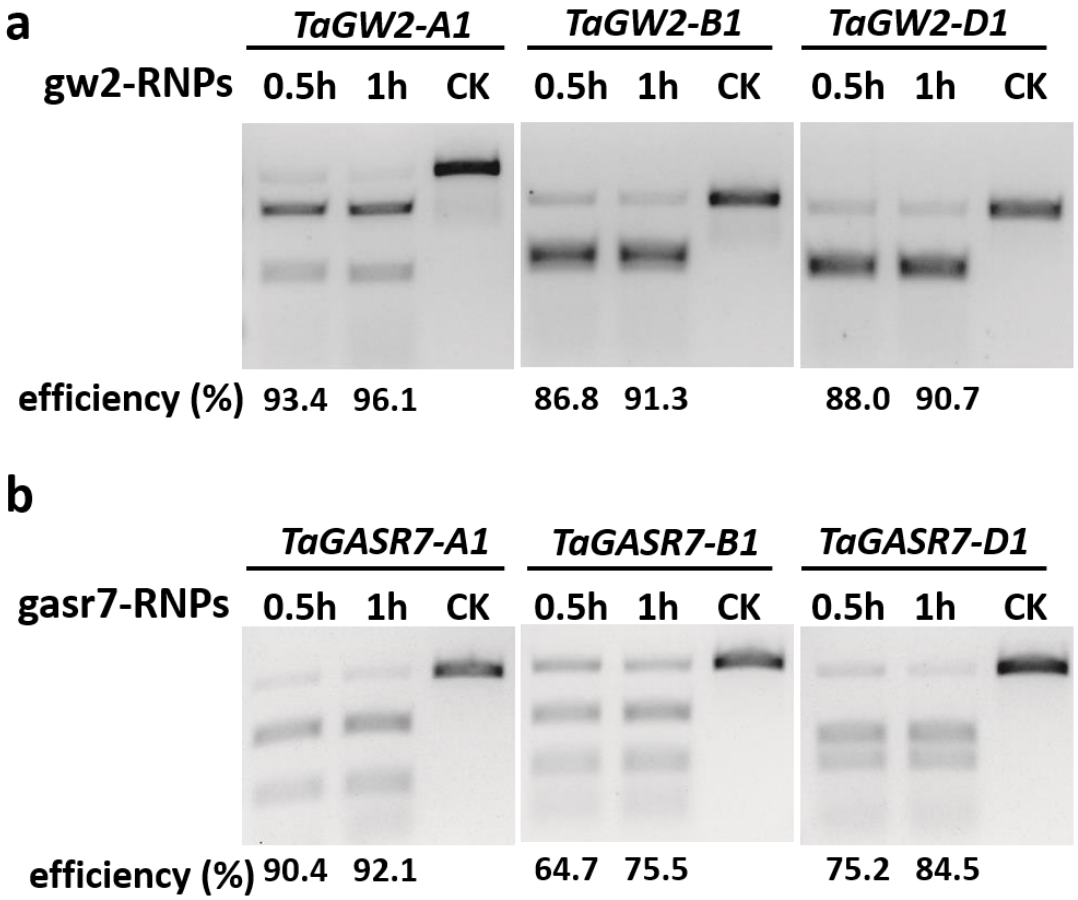
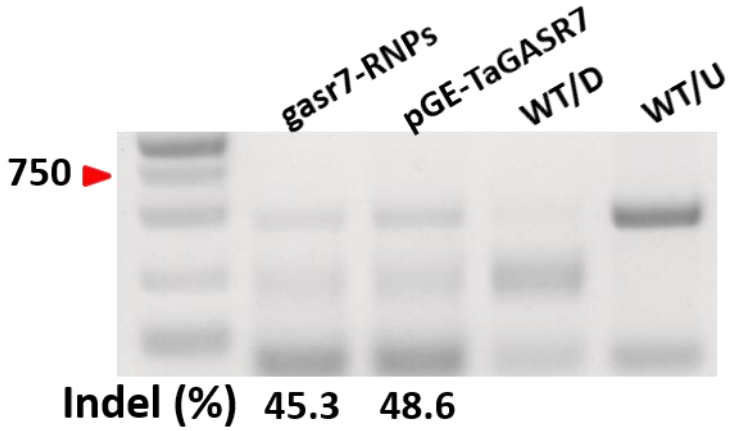
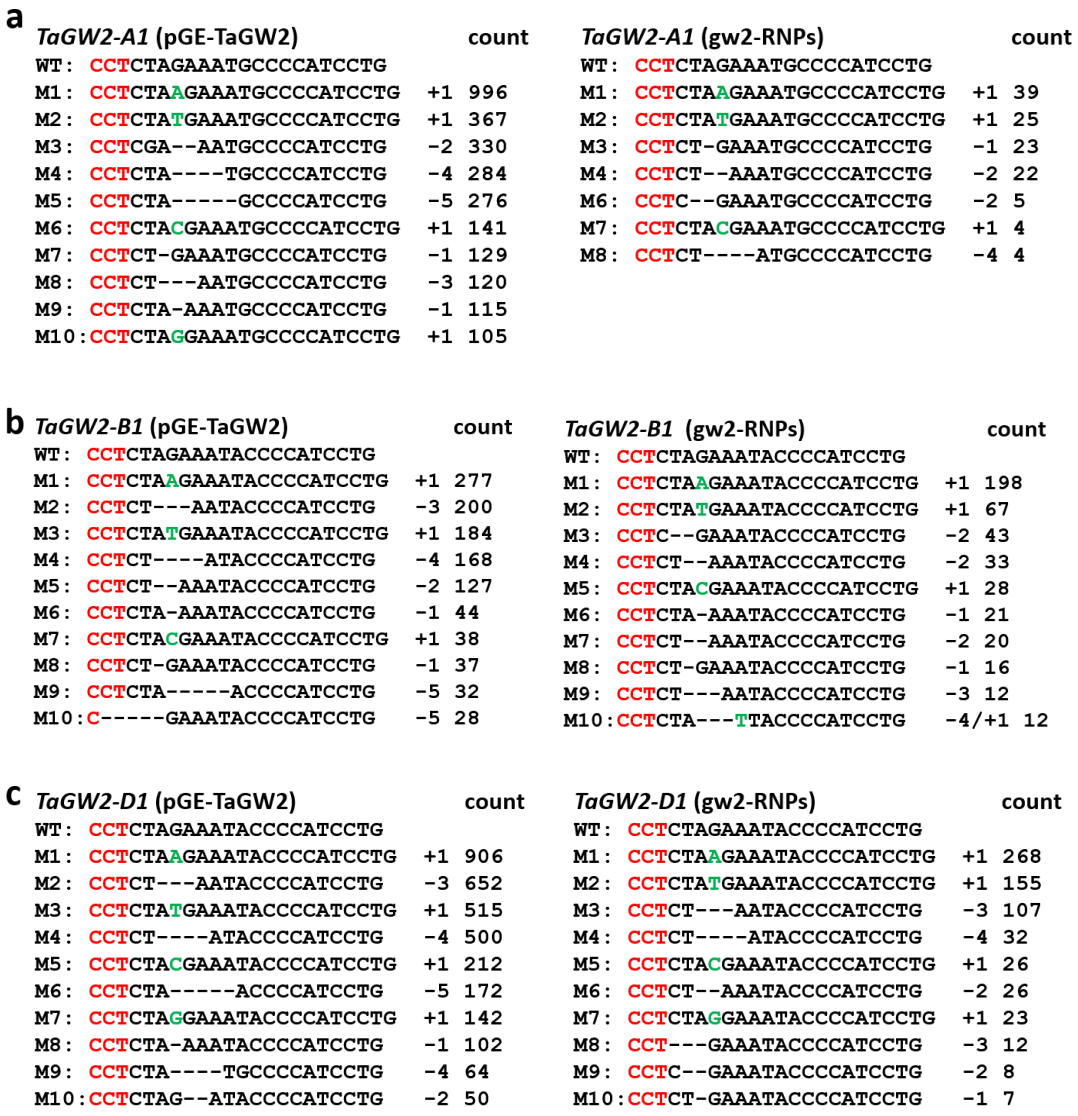


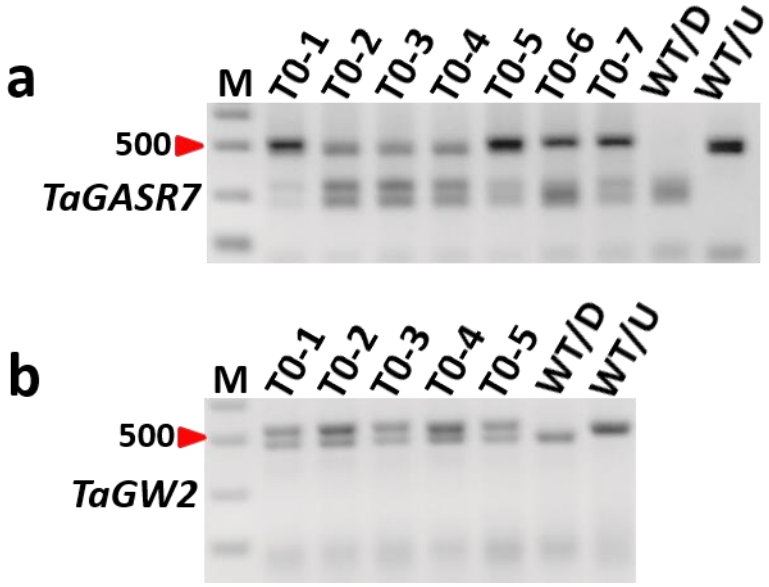
Supplementary Figure 1. *In vitro* cleavage of three homoeologs of *TaGW2* (*TaGW2-A1, -B1* and *-D1*) and *TaGASR7* (*TaGASR7-A1, -B1* and *-D1*) using gw2-RNPs or gasr7-RNPs. CK indicates the incubation without the addition of RNPs.



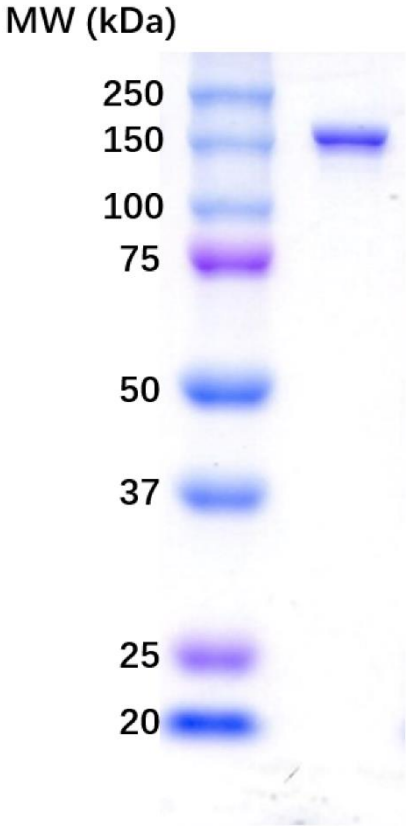
Supplementary Figure 2. Mutagenesis frequencies of *TaGASR7* (induced by gasr7-RNPs or pGE-TaGASR7) in wheat protoplasts analyzed by PCR-RE assay. WT/D and WT/U indicate wild type PCR amplicons with or without restriction enzyme digestion.



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-PSGE image of purified Cas9 protein. 1 μl 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
TaGW2	CCTCTAGAAATACCCCATCCTG	CTTGCAGGATGGGGTATTTCTAG	AAACCTAGAAATACCCCATCCTG	Deep sequencing and PCR-RE XbaI
TaGASR7	CCGCCGGGCACCTACGGCAAC	CTTGGTTGCCGTAGGTGCCCGG	AAACCCGGGCACCTACGGCAAC	Deep sequencing and PCR-RE BcnI

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 (%)
TaGW2-A1	pGE-TaGW2	425527	3245	0.76
	gw2-RNPs	390295	122	0.03
	Control	408883	10	0
TaGW2-B1	pGE-TaGW2	133533	1320	0.99
	gw2-RNPs	296538	541	0.18
	Control	220458	21	0.01
TaGW2-D1	pGE-TaGW2	433865	4333	1.00
	gw2-RNPs	323010	677	0.21
	Control	213139	19	0.01
TaGASR7	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	ATGCCAACCCTTGCGTGTGCGT TCCTGCTTGTGGGAGCTTTATG	Amplifying the *TaGW2* target site
GW2-A1-F	CTGCCATTACTTTGTATTTTGGTAATA	Amplifying the *TaGW2-A1* target site and 1st PCR for deep sequencing
GW2-B1-F	GTTCAGATGGCAATCTAAAAGTT	Amplifying the *TaGW2-B1* target site and 1st PCR for deep sequencing
GW2-D1-F	GCATGTACTTTGATTGTTTGCGTGA	Amplifying the T*aGW2-D1* target site and 1st PCR for deep sequencing
GW2-A1/B1/D1-R	TCCTTCCTCTCTTACCACTTCCC	Amplifying the *TaGW2-A1, -B1 and -D1* target site and 1st 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 *TaGASR7* target site and 1st PCR for deep sequencing
GASR7-A1/B1/D1-F	CCTTCATCCTTCAGCCATGCAT	Amplifying the *TaGASR7-A1, -B1 and -D1* target site
GASR7-A1-R	CCACTAAATGCCTATCACATACG	Amplifying the *TaGASR7-A1* target site
GASR7-B1-R	AGGGCAATTCACATGCCACTGAT	Amplifying the *TaGASR7-B1* target site
GASR7-D1-R	CCTCCATTTTTCCACATCTTAGTCC	Amplifying the *TaGASR7-D1* target site
BA1-GW2-AF BA2-GW2-AR	CGATGTTGCCTTTTGAGCAACCAACG TGACCATCCATGCTTGATGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-A1* of pGE-TaGW2
BA3-GW2-BF BA4-GW2-BR	ACAGTGTGCCTTTTGAGCAACCAACG GCCAATTCCATGCTTGATGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-B1* of pGE-TaGW2
BA5-GW2-DF BA6-GW2-DR	CAGATCTGCCTTTTGAGCAACCAACG CTTGTATCCATGCTTGCTGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-D1* of pGE-TaGW2
BA13-GW2-AF BA14-GW2-AR	AGTCAATGCCTTTTGAGCAACCAACG AGTTCCTCCATGCTTGATGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-A1* of gw2-RNPs
BA15-GW2-BF BA16-GW2-BR	ATGTCATGCCTTTTGAGCAACCAACG CCGTCCTCCATGCTTGATGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-B1* of gw2-RNPs
BA17-GW2-DF BA18-GW2-DR	GTAGAGTGCCTTTTGAGCAACCAACG GTCCGCTCCATGCTTGCTGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-D1* of gw2-RNPs
BA43-GW2-AF BA44-GW2-AR	TACAGCTGCCTTTTGAGCAACCAACG TATAATTCCATGCTTGATGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-A1* of WT
BA45-GW2-BF BA46-GW2-BR	TCATTCTGCCTTTTGAGCAACCAACG TCCCGATCCATGCTTGATGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-B1* of WT
BA47-GW2-DF BA48-GW2-DR	TCGAAGTGCCTTTTGAGCAACCAACG TCGGCATCCATGCTTGCTGGCTGGTG	2nd PCR for deep sequencing of *TaGW2-D1* of WT
BA41-GASR7-F BA42-GASR7-R	GCGCTAGAAGGCGTGCCTGACCTACT TAATCGCAAGAATCTAGGGGCACTTGG	2nd PCR for deep sequencing of *TaGASR7* of WT
BA43-GASR7-F BA44-GASR7-R	TACAGCGAAGGCGTGCCTGACCTACT TATAATCAAGAATCTAGGGGCACTTGG	2nd PCR for deep sequencing of *TaGASR7* of gasr7-RNPs

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

Plant ID	Genotype of *TaGW2-A1*	Genotype of *TaGW2-B1*	Genotype of *TaGW2-D1*
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	CCTCTAGAAATACCCCATCCTG			
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