

Supplementary Materials

Supplementary Table S1. Primers used in this study.

| Primer Name | Base Sequence (5'→3') | Purpose |
|-------------|--|--|
| 22KT1-F | GGCACATGGCGCGCACGCACGTA | Construction of 22KT1 target site |
| 22KT1-R | AAACTACGTGCGTGCGCGCCATG | Construction of 22KT1 target site |
| 22KT2-F | GCCGCGCCATGTCGTCCGCCGCT | Construction of 22KT2 target site |
| 22KT1-R | GGCACATGGCGCGCACGCACGTA | Construction of 22KT2 target site |
| 22T3-F | GGCACACGTACCACCAAATGCTA | Construction of 22T3 target site |
| 22T3-R | AAACTAGCATTGTTGGTGGTACGTG | Construction of 22T3 target site |
| U-F | AAACTACGTGCGTGCGCGCCATG | U#-T-gRNA expression-box first PCR |
| gRNA-R | GCCGCGCCATGTCGTCCGCCGCT | U#-T-gRNA expression-box first PCR |
| B1' | TTCAGAGGTCTCTCTCGCACTGGAA TCGGCAGCAAAGG | U#-T-gRNA expression-box second PCR |
| B2 | AGCGTGGGTCTCGTCAGGGTCCATC CACTCCAAGCTC | U#-T-gRNA expression-box second PCR |
| B2' | TTCAGAGGTCTCTCTGACACTGGAA TCGGCAGCAAAGG | U#-T-gRNA expression-box second PCR |
| BL | AGCGTGGGTCTCGACCGGGTCCATC CACTCCAAGCTC | U#-T-gRNA expression-box second PCR |
| SP1 | CCCGACATAGATGCAATAACTTC | CRISPR/Cas9 expression-vector verification |
| SP2 | GCGCGGTGTCATCTATGTTACT | CRISPR/Cas9 expression-vector verification |
| HPT-F1 | CTTCTGCGGGCGATTTGT | Transgenic plant detection |
| HPT-R1 | CAGCGTCTCCGACCTGAT | Transgenic plant detection |
| Cas9-F1 | GTTGGTATTACGGGGTGCCT | Transgenic plant detection |
| Cas9-R1 | CGACGATGTTGCCGAAGATGG | Transgenic plant detection |
| RR-CJ3F | GGATCAGCTGTTGTGTAAAAATGTC | 22KT12,22T3 target mutation detection |
| RR-CJ3R | CTTGCAACATTTTCCCTGGTGAG | 22KT12,22T3 target mutation detection |

Supplementary Table S2. CRISPR/Cas9-induced mutations in *OsRR22* and Transgene-free ratio in T1 generation.

| T0 Plant | Target Site | Host Cultivar | No. of Plants Examined | Transgene-Free Ratio (%) in T1 |
|----------|-------------|---------------|------------------------|--------------------------------|
| T1430 | 22KT12 | 733B | 18 | 33.33 (6/18) |
| T1431 | 22KT12 | 733B | 18 | 27.78 (5/18) |
| T1432 | 22KT12 | 733B | 18 | 0 (0/18) |
| T1447 | 22KT12 | 733B | 18 | 16.67 (3/18) |
| T1448 | 22KT12 | 733B | 18 | 22.23 (4/18) |
| T1347 | 22T3 | HZ | 18 | 11.12 (2/18) |
| T1348 | 22T3 | HZ | 18 | 0 (0/18) |
| T1349 | 22T3 | HZ | 18 | 27.78 (5/18) |
| T1350 | 22T3 | HZ | 18 | 27.78 (5/18) |
| T1351 | 22T3 | HZ | 18 | 16.67 (3/18) |

Supplementary Table S3. Mutation detections in the putative CRISPR/Cas9 off-target sites.

| Target Site | Putative Off-Target Site | Putative Off-Target Locus | Sequence of Putative Off-Target Site ¹ | No. of Mismatching Bases | No. of Plants Detected | No. of Plants with Mutations |
|-------------|--------------------------|---------------------------|---|--------------------------|------------------------|------------------------------|
| 22KT1 | off-22KT1-1 | Chr11:1220469-1220488 | TGATTAAAGAACCACAGAA <u>AGG</u> | 4 | 90 | 0 |
| 22KT1 | off-22KT1-2 | Chr11:14999083-14999102 | TTCTTAGAAGAATCACAGGT <u>AGG</u> | 4 | 90 | 0 |
| 22KT1 | off-22KT1-3 | Chr11:4021308-4021327 | TGCTCAGAAGATCCACAGTT <u>AGG</u> | 3 | 90 | 0 |
| 22KT1 | off-22KT1-4 | Chr11:8539864-8539883 | TTAGCAGCAGCACCACAGGT <u>TGG</u> | 4 | 90 | 0 |
| 22KT2 | off-22KT2-1 | Chr3:35790995-35791014 | CTGGGCTCTTATGCAGCAGA <u>TGG</u> | 4 | 90 | 0 |
| 22KT2 | off-22KT2-2 | Chr3:12627682-12627701 | CCTCTCTTCTTTGCAGCTGA <u>TGG</u> | 4 | 90 | 0 |
| 22T3 | off-22T3-1 | Chr7:22781210-22781229 | TGACAATGAATCTGATGAAG <u>AGG</u> | 4 | 90 | 0 |
| 22T3 | off-22T3-2 | Chr1:33054780-33054799 | TGACCAAGGACGTGATGAAG <u>GGG</u> | 5 | 90 | 0 |
| 22T3 | off-22T3-3 | Chr1:32373735-32373754 | AGTCAAAGACTGTGAGGAAA <u>GGG</u> | 4 | 90 | 0 |
| 22T3 | off-22T3-4 | Chr1:32373735-32373754 | AGACCAA ¹ ACTGTATTGAAG <u>GGG</u> | 4 | 90 | 0 |

¹The base that matched the sgRNA are marked in black. The base that mismatched the sgRNA are marked in red. The sequences of the PAM are underlined.