DNA-free CRISPR-Cas9 gene editing of wild tetraploid tomato *Solanum peruvianum* using protoplast regeneration

Choun-Sea Lin[1,*], Chen-Tran Hsu[1], Yu-Hsuan Yuan[1], Po-Xing Zheng[1,2], Fu-Hui Wu[1], Qiao-Wei Cheng[1], Yu-Lin Wu[1,2], Ting-Li Wu[1,2], Steven Lin[3], Jin-Jun Yue[4], Ying-Huey Cheng[5], Shu-I Lin[6], Ming-Che Shih[1,*], Jen Sheen[7,*], Yao-Cheng Lin[1,2,*,+]

[1] Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

[2] Biotechnology Research Center in Southern Taiwan, Academia Sinica, Tainan, Taiwan

[3] Institute of Biochemistry, Academia Sinica, Taipei, Taiwan

[4] Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou, China

[5] Plant Pathology Division, Taiwan Agricultural Research Institute, Taichung, Taiwan

[6] Department of Horticulture and Landscape Architecture, National Taiwan University, Taipei, Taiwan

[7] Department of Molecular Biology and Centre for Computational and Integrative Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA 02114, USA

*Senior authors: cslin99@gate.sinica.edu.tw; mcshih@gate.sinica.edu.tw; sheen@molbio.mgh.harvard.edu; yalin@sinica.edu.tw

+Corresponding author:

Yao-Cheng Lin

Biotechnology Research Center in Southern Taiwan, Academia Sinica
No 100. Guiren 13th Road, Sec. 1, Guiren, Tainan City, 711010,Taiwan
Tel: +886 6 303 2312
Email: yalin@sinica.edu.tw

ORCID IDs: 0000-0001-9566-2952 (C-SL); 0000-0001-5827-5865 (P-XZ); 0000-0002-7590-793X (M-CS); 0000-0002-9390-795X (Y-CL);

Key words: Virus resistant, DNA-free, microRNA synthesis, Ribonucleoprotein, peptide hormone

Supplemental Data

Supplemental Figure S1. Effects of cytokinins on callus induction (1st subculture) and callus proliferation (2nd subculture).

Supplemental Figure S2. Effects of cytokinins on callus in solid medium (3rd subculture).

Supplemental Figure S3. Flow cytometric analysis of the nuclear DNA contents of tetraploid plants regenerated from *SpProSys* RNP-transfected protoplasts.

Supplemental Figure S4. Phenotypes of diploid and tetraploid plants regenerated from protoplasts transfected with CRISPR reagents.

Supplemental Figure S5. Progeny analysis of *SpSGS3*.

Supplemental Figure S6. Progeny analysis of *SpRDR6*.

Supplemental Figure S7. Progeny analysis of *SpPR-1*.

Supplemental Figure S8. Illumina sequencing coverage for the tomato SL4.0 genome assembly.

Supplemental Figure S9. Phenotypes of the *spsgs3* and *sprdr6* null mutants.

Supplemental Figure S10. Symptoms and TYLCV proliferation on *in vitro*-cultured *S. peruvianum* plants inoculated with the infectious TYLCV clone.

Supplemental Table S1. *SpSGS3* gene sequences of the *SpSGS3* mutants.

Supplemental Table S2. *SpRDR6* gene sequences of the *SpRDR6* mutants.

Supplemental Table S3. *SpPR-1* gene sequences of the *SpPR-1* mutants.

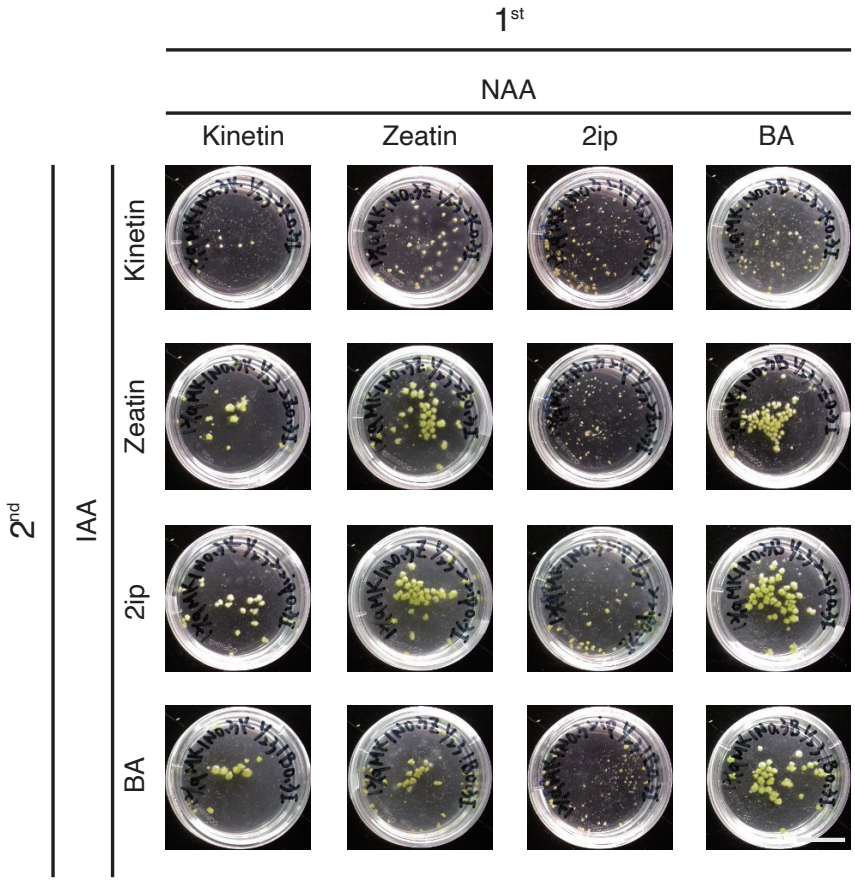
Supplemental Table S4. *SpProSys* gene sequences of the *SpProSys* mutants.

Supplemental Table S5. *SpMlo1* gene sequences of the *SpMlo1* mutants.

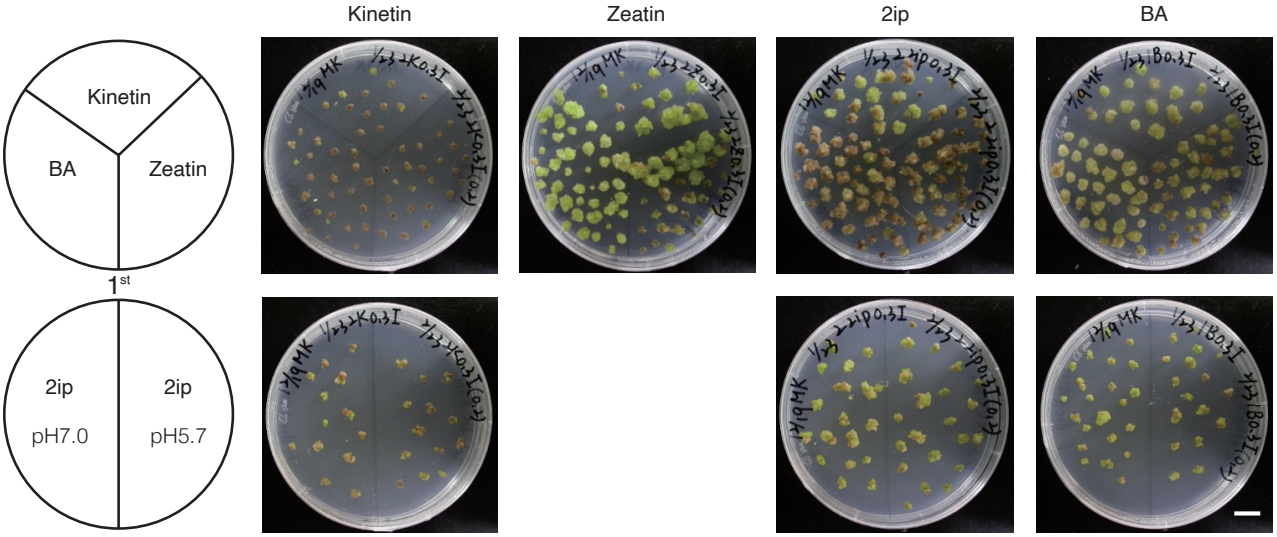
Supplemental Table S7. Overview of Illumina WGS sequencing, mapping rate and SRA number.

Supplemental Table S8. Primers used in these studies.

Supplemental Table S9. Locus ID, orthologs in *S. lycopersicum* and the cDNA sequence used in this study.

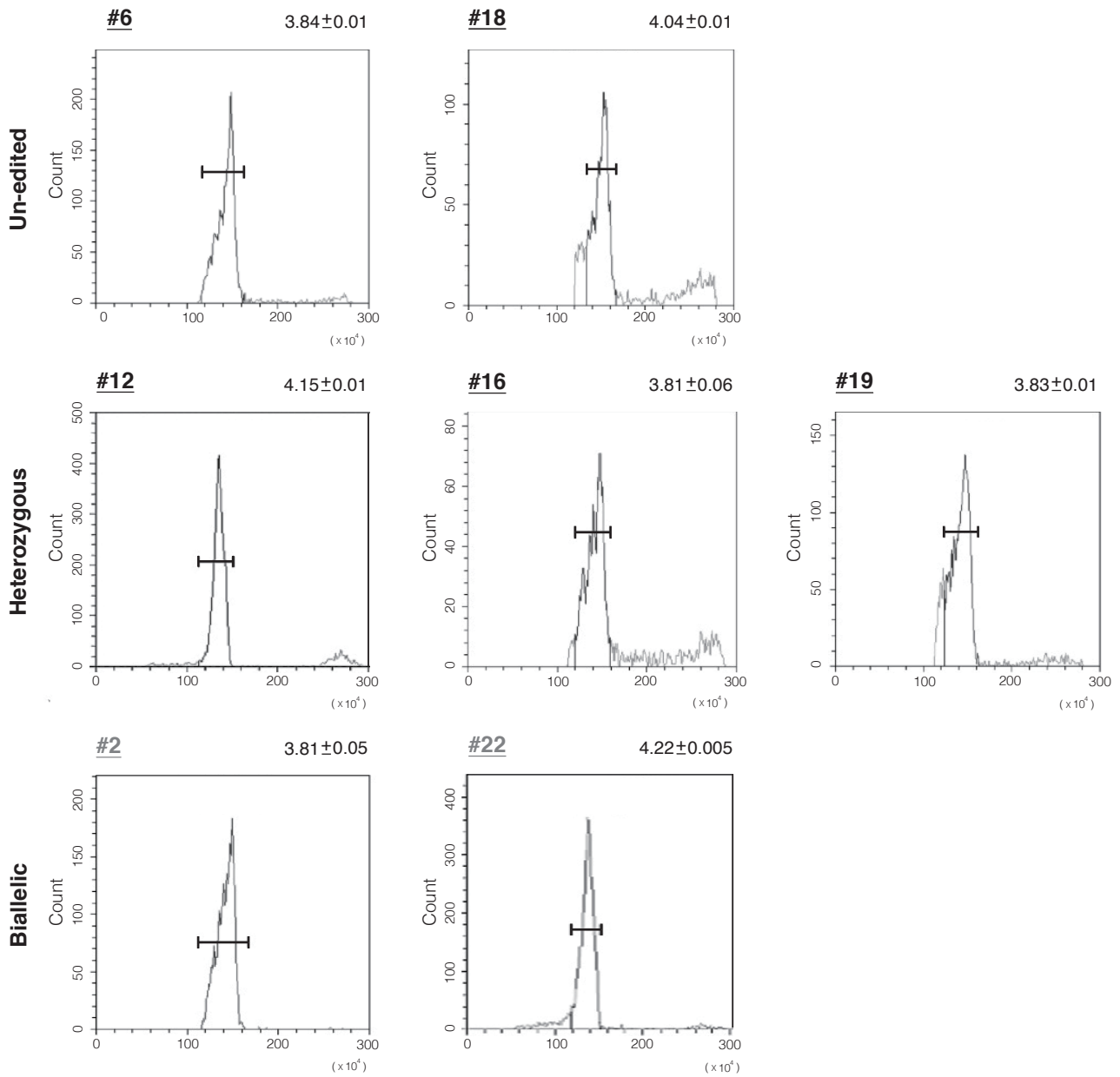


Supplemental Figure S1. Effects of cytokinins on callus induction (1st subculture) and callus proliferation (2nd subculture).
The effects of cytokinins [kinetin, zeatin, 6-(γ,γ-Dimethylallylamino)purine (2ip), and 6-Benzylaminopurine (BA)] during these two stages were investigated separately. Different cytokinins were added during callus induction [1st subculture, 1/2 Murashige and Skoog (MS) medium supplemented with 3% (v/v) sucrose, 0.4 M mannitol, pH 5.7 liquid medium supplemented with 0.2 mg/L cytokinin and 1 mg/L 1-Naphthaleneacetic acid (NAA)]. Kinetin yielded the fewest calli, and the three other cytokinins led to better callus induction. During callus proliferation [2nd subculture, 1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, pH 5.7 liquid medium supplemented with 2 mg/L cytokinin and 0.3 mg/L Indole-3-acetic acid (IAA)], the addition of zeatin, 2ip, and BA caused the callus to grow and turn green. Inclusion of 2ip during callus induction yielded the same number of cells as the other cytokinin treatments, but the cell clusters were smaller and did not grow easily when directly transferred to callus proliferation medium in the light. Therefore, zeatin and BA are the best treatments for liquid culture. Bar = 1 cm.

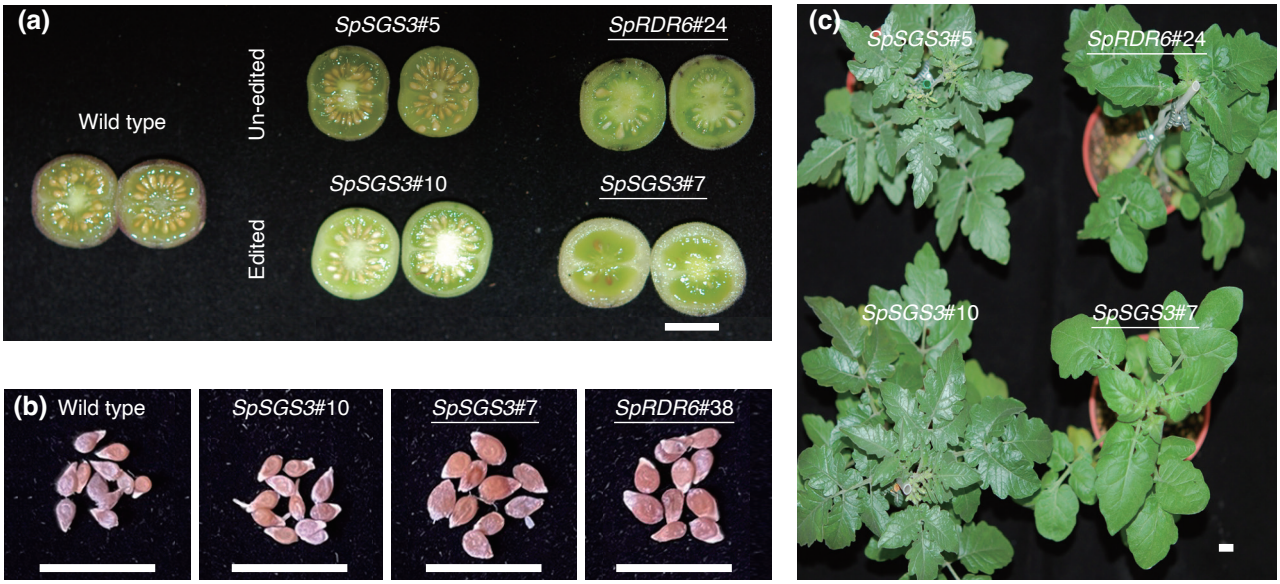


Supplemental Figure S2. Effects of cytokinins on callus in solid medium (3rd subculture).

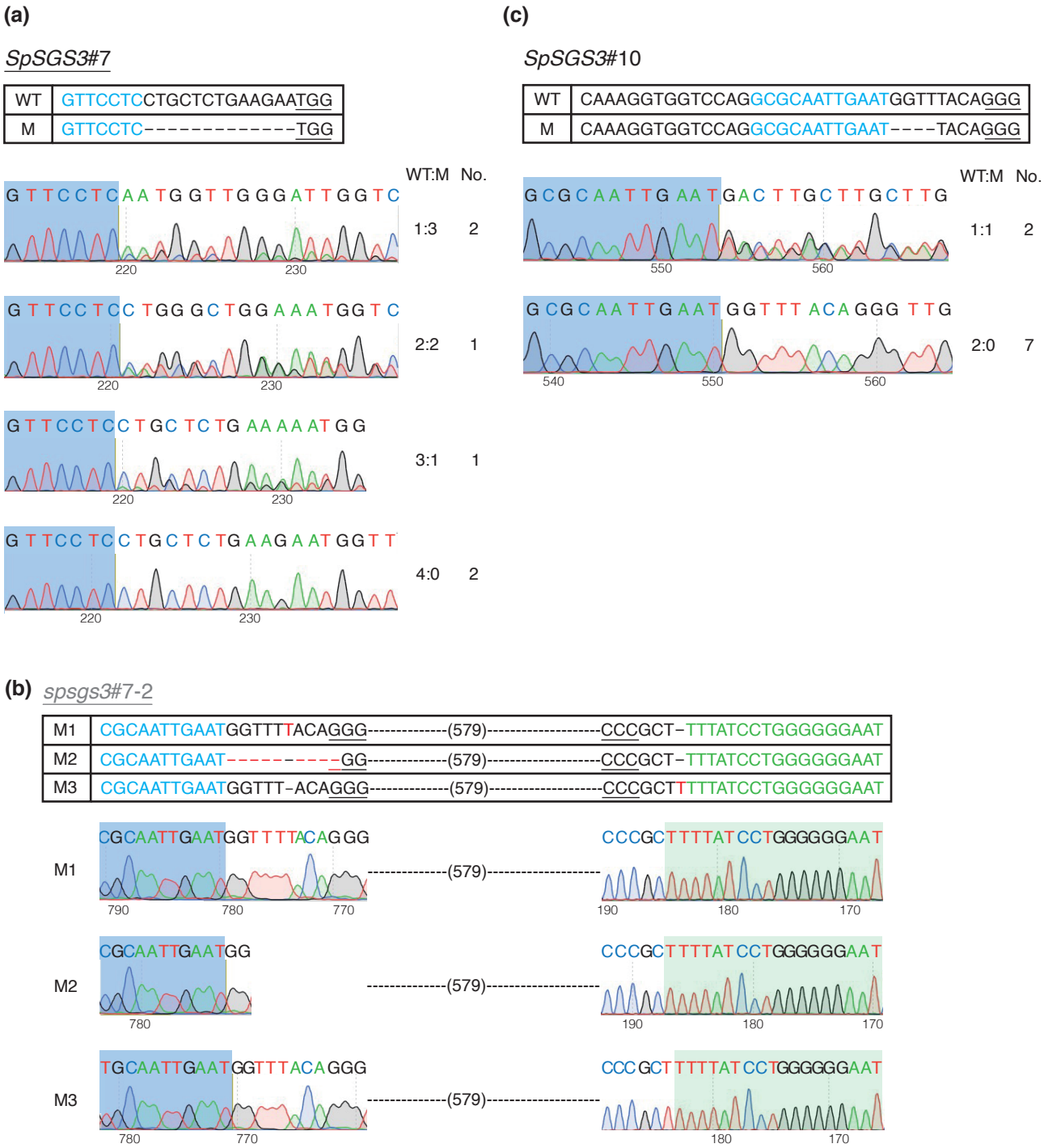
Calli from media containing different cytokinins (2nd subculture) were transferred to solid medium containing the same cytokinin (3rd subculture). Cytokinin in the medium had a strong effect on callus growth (Figure S4). Regardless of the callus induction medium used, browning of the callus occurred in solid medium supplemented with kinetin. Callus derived from 2ip callus induction medium proliferated only in 2ip solid medium. BA and zeatin had similar effects on callus growth, but calli on zeatin medium showed more greening. We therefore identified zeatin as the most suitable cytokinin for use in solid medium. Bar = 1 cm.



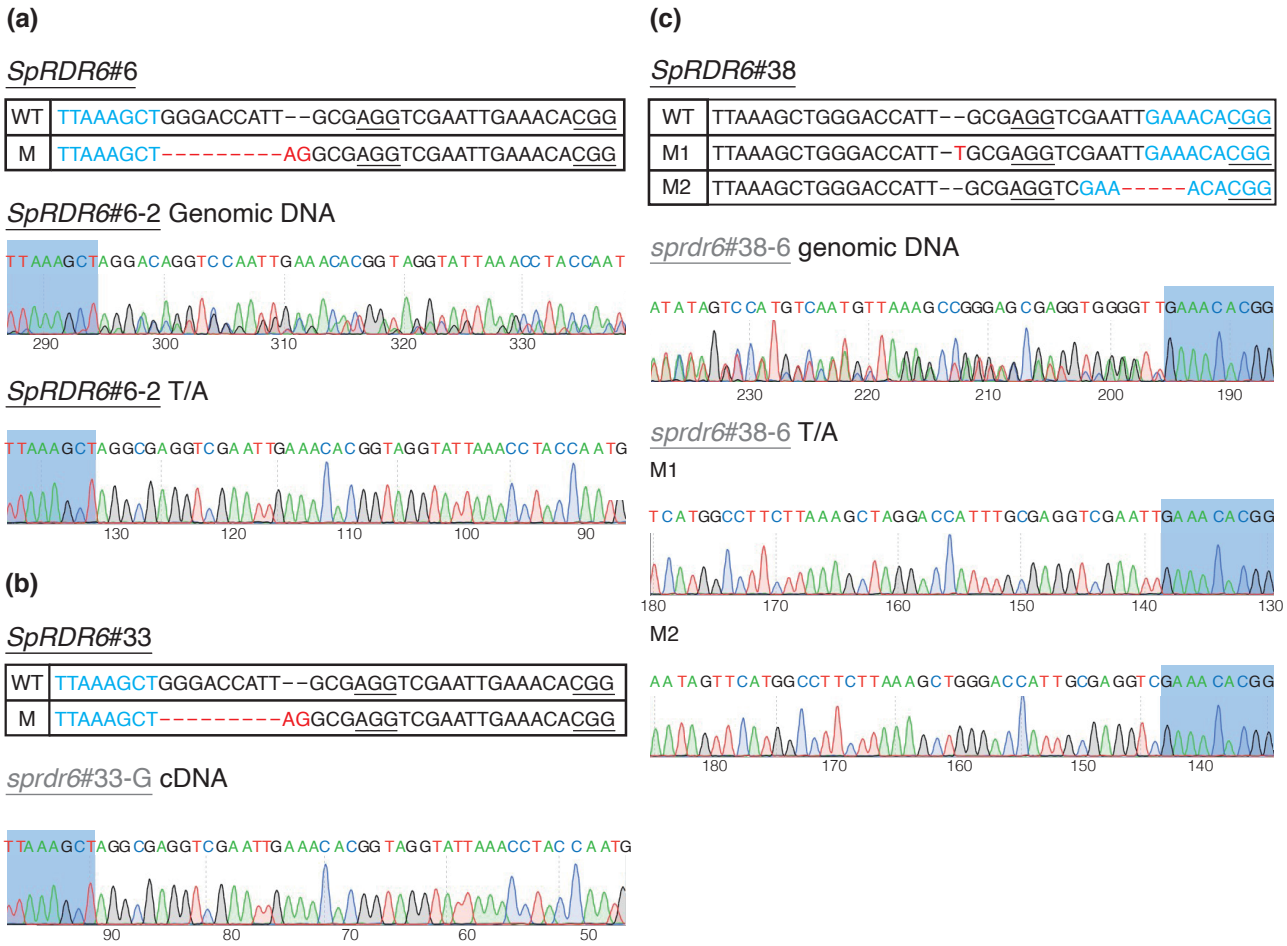
Supplemental Figure S3. Flow cytometric analysis of the nuclear DNA contents of tetraploid plants regenerated from *SpProSys* RNP-transfected protoplasts.
The number of regenerated plants is shown at the top left of each panel. Gray font: null mutant. The genome sizes are shown at the top right. The results are derived from three technical repeats. Unit: picogram (pg). Un-edited: The *SpProSys* sequences are similar to the wild type. Chicken erythrocyte nuclei (CEN: 2.5 Gb) were used as the calibration standard. The bar indicates the area used to count nuclei. The genome sizes of all seven regenerants were measured by flow cytometry, including two un-edited, three heterozygous, and two biallelic plants that were tetraploid. Both tetraploid and diploid regenerants derived from *SpProSys* RNP transfections flowered normally, and no distinctive phenotype was observed.



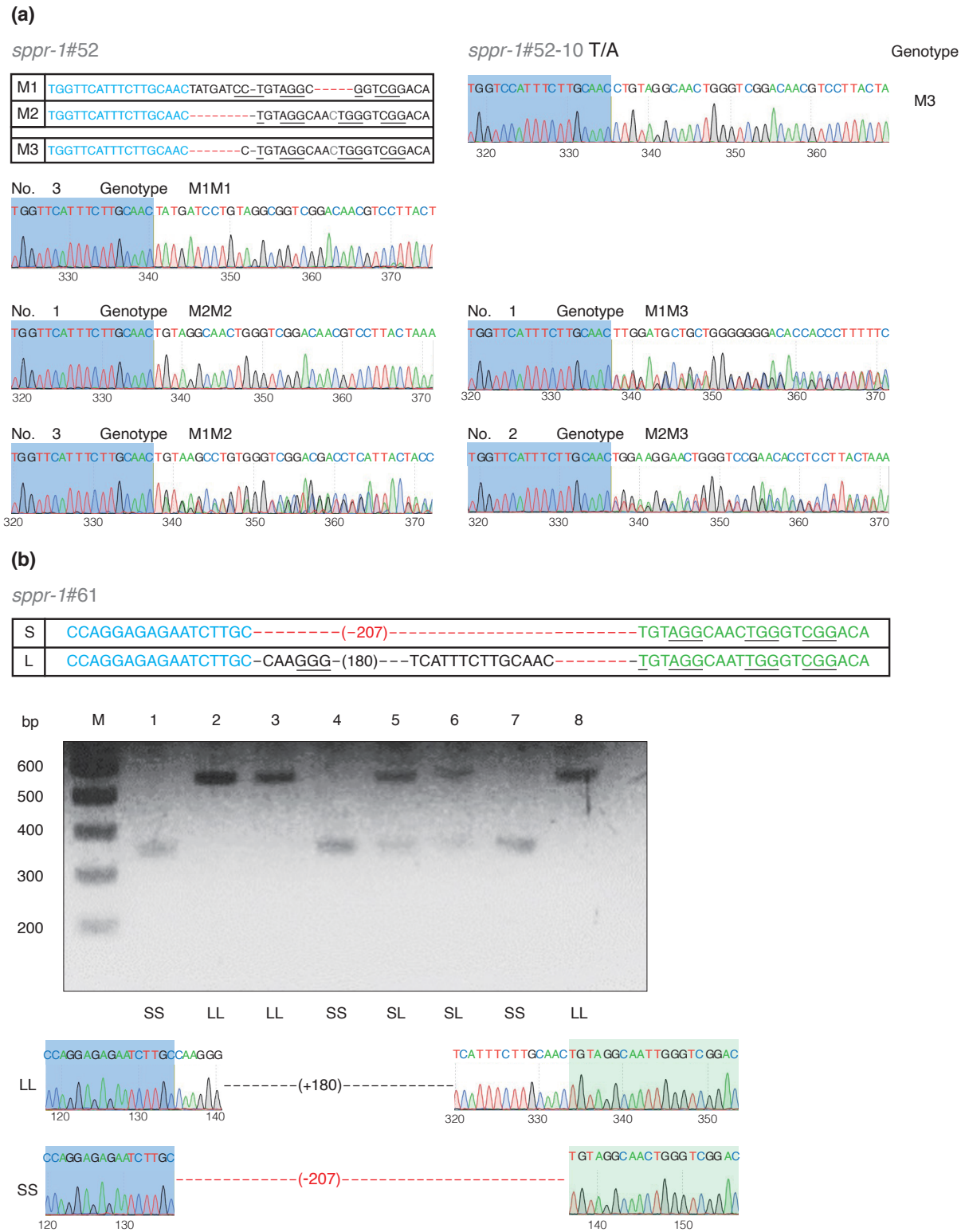
Supplemental Figure S4. Phenotypes of diploid and tetraploid plants regenerated from protoplasts transfected with CRISPR reagents.
Underline: 4*n*. Bars = 1 cm. *SpSGS3*#10, *SpSGS3*#7 and *SpRDR6*#38 contained mutated alleles. (a) the fruits of diploid and tetraploids regenerated from transfected protoplasts. (b) T_1 seeds of the heterozygous diploid (*SpSGS3*#10) and tetraploid (*SpSGS3*#7 and *SpRDR6*#38) mutants. (c) 1.5-month-old T_1 seedling derived from T_0 transfected protoplast regenerated plants.



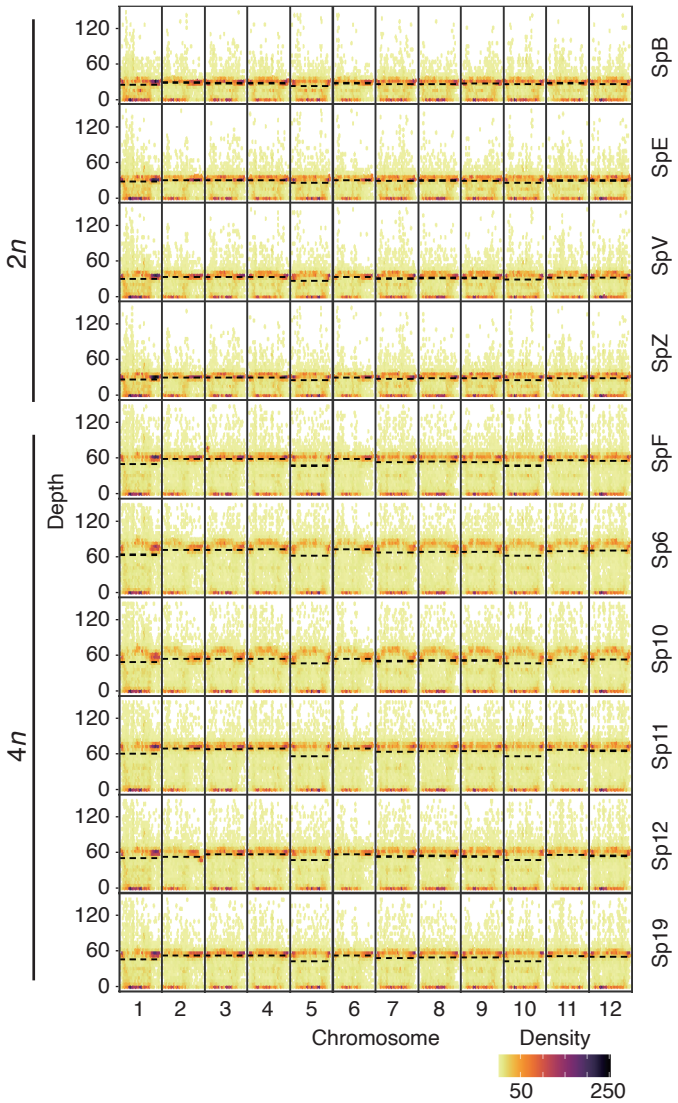
Supplemental Figure S5. Progeny analysis of *SpSGS3*.
Underlined regenerated plant name: tetraploid. Red font: mutated nucleotide. Green/blue font: sequences shown in the green/blue boxes in the Sanger sequencing results. WT: wild type. M: mutant. WT:M: wild type/mutant ratio based on Sanger sequencing results. No.: number of progeny in this ratio. (a) *SpSGS3*#7 T1 progeny analysis. The allele sequences in the GTTCCTCCTGCTCTGAAGAA target site are listed; 0–3 mutated alleles were identified. This regenerated plant was shown to be allotetraploid. (b) The PCR product of the *spsgs3*#7-2 null mutant was subjected to T/A cloning, and the clones were subjected to Sanger sequencing. Three types of mutated alleles were identified. (c) Analysis of diploid *SpSGS3*#10 T_1 progeny.



Supplemental Figure S6. Progeny analysis of *SpRDR6*.
Underlined regenerated plant name: tetraploid. Red font: mutated nucleotides. Blue font: sequences shown in blue boxes in the Sanger sequencing results. (a) *SpRDR6*#6-2 genotyping. Top: allele sequences. Middle: The Sanger sequencing results indicate the presence of multiple peaks after TTAAGCT. Bottom: The T/A cloning results demonstrate that *SpRDR6*#6-2 contains a mutated allele (M) similar to *SpRDR6*#6. (b) RT-PCR product of the *sprdr6*#33-G null mutant. The result indicates that *sprdr6*#33-G is a homozygous null mutant. The mutated allele can still generate a transcript. (c) Genotyping of the *sprdr6*#38-6 null mutant. Top: The allele sequences of *SpRDR6*#38. Middle: Sanger sequencing results of *sprdr6*#38-6 genomic DNA. Bottom: The M1 and M2 mutated alleles identified by T/A cloning without wild-type alleles.

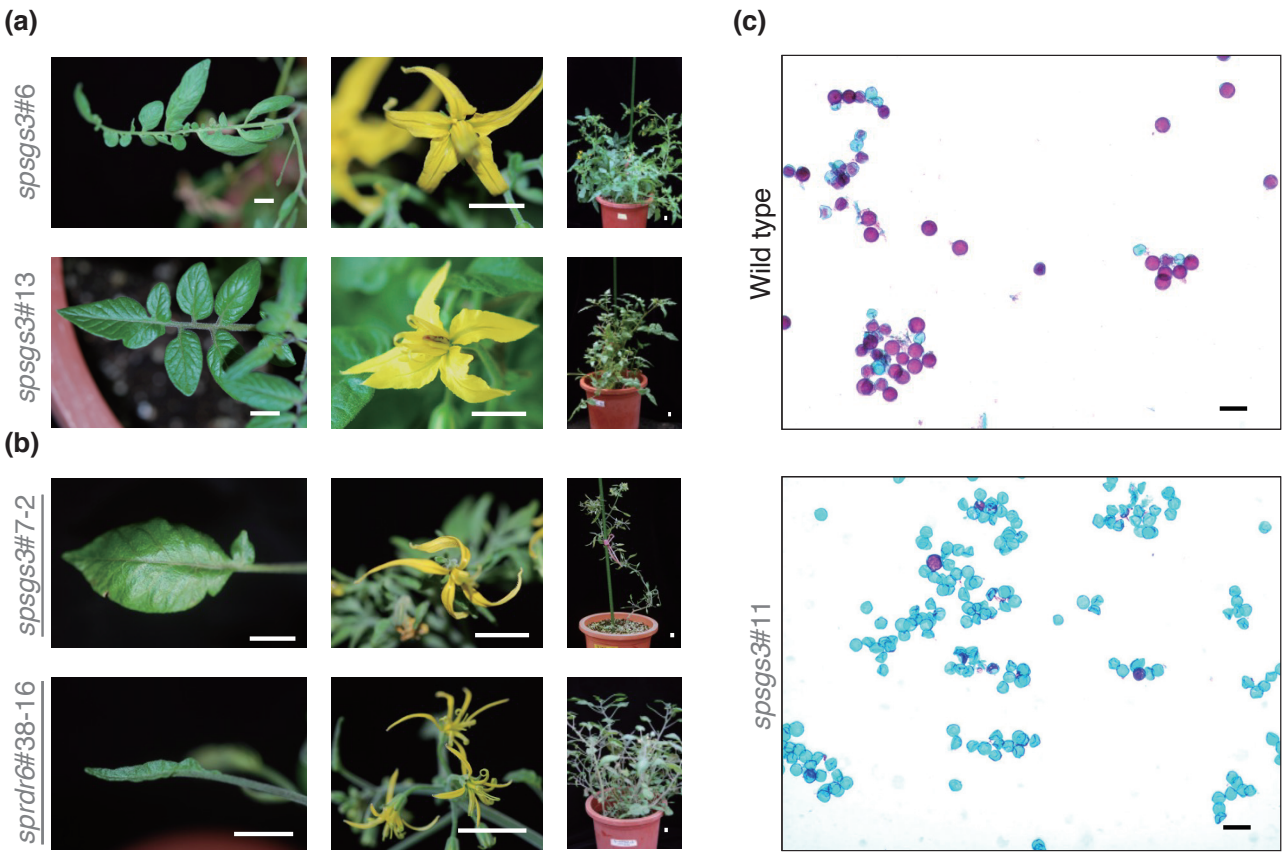


Supplemental Figure S7. Progeny analysis of *SpPR-1*.
Underlined regenerated plant name: tetraploid. Red font: mutated nucleotide(s). Blue font: sequences shown in blue boxes in the Sanger sequencing results. (a) Progeny analysis of *sppr-1*#52. Top: allele sequences. Middle: Sanger sequencing results of different genotypes. Multiple peaks are shown in heterozygous lines (M1M2, M1M3, M2M3). No.: number of progeny of each genotype. Bottom: M3 sequence identified by T/A cloning. (b) Progeny analysis of *sppr-1*#61. Top: allele sequences. Middle: *SpPR-1* genomic PCR products of *sppr-1*#61 progeny. The genotypes of individual progeny were determined based on DNA size and are shown below the image. Sanger sequencing results for the LL and SS genotypes.

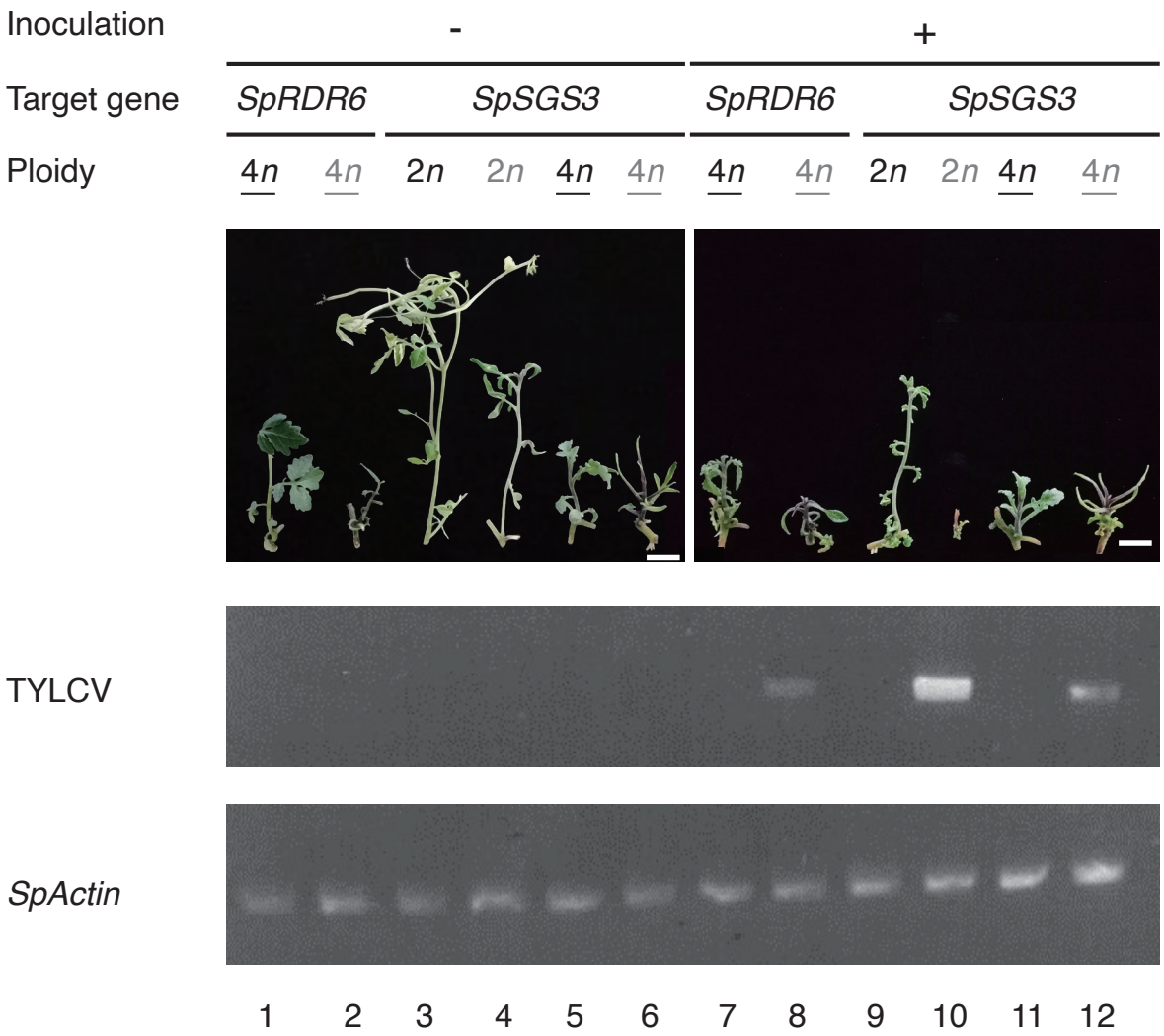


Supplemental Figure S8. Illumina sequencing coverage for the tomato SL4.0 genome assembly.

The Illumina PE reads were mapped by BWA and the sequencing depth was calculated in 10kb window size. Coverage is plotted using 30 bins per chromosome on the X axis. Black dashed line: median of the sequencing coverage of each chromosome.



Supplemental Figure S9. Phenotypes of the *spsgs3* and *sprdr6* null mutants. Underlined regenerated plant name: tetraploid. (a) Wiry phenotypes of T_0 diploid *spsgs3* null mutants #6 and #13. Bar = 1 cm. (b) Wiry phenotypes of T_1 tetraploid *spsgs3*#7-2 and *sprdr6*#38-16. Bar = 1 cm. (c) Alexander staining of wild-type and *spsgs3*#11 pollen. Bar = 50 µm.



Supplemental Figure S10. Symptoms and TYLCV proliferation on *in vitro*-cultured *S. peruvianum* plants inoculated with the infectious TYLCV clone.
Gray: null mutant. Underline: 4*n*. Bars = 1 cm. *SpRDR6*#2 and *SpSGS3*#24 were non-mutated protoplast regenerated plants. Line 1, 7: *SpRDR6*#2; 2, 8: *sprdr6*#38-6; 3, 9: Wild type; 4, 10: *spsgs3*#11; 5, 11: *SpSGS3*#24; 6, 12: *spsgs3*#7-2