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Research Article

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

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Abstract

Wild tomatoes (*Solanum peruvianum*) are important genomic resources for tomato research and breeding. Development of a foreign DNA-free clustered regularly interspaced short palindromic repeat (CRISPR)-Cas delivery system has potential to mitigate public concern about genetically modified organisms. Here, we established a DNA-free CRISPR-Cas9 genome editing system based on an optimized protoplast regeneration protocol of *S. peruvianum*, an important resource for tomato introgression breeding. We generated mutants for genes involved in small interfering RNAs biogenesis, *RNA-DEPENDENT RNA POLYMERASE 6* (*SpRDR6*), and *SUPPRESSOR OF GENE SILENCING 3* (*SpSGS3*); pathogen-related peptide precursors, *PATHOGENESIS-RELATED PROTEIN-1* (*SpPR-1*) and *PROSYSTEMIN* (*SpProSys*); and fungal resistance (*MILDEW RESISTANT LOCUS O, SpMlo1*) using diploid or tetraploid protoplasts derived from *in vitro*-grown shoots. The ploidy level of these regenerants was not affected by PEG-Ca^{2+}-mediated transfection, CRISPR reagents, or the target genes. By karyotyping and whole genome sequencing analysis, we confirmed that CRISPR-Cas9 editing did not introduce chromosomal changes or unintended genome editing sites. All mutated genes in both diploid and tetraploid regenerants were heritable in the next generation. *spsgs3* null T0 regenerants and *sprdr6* null T1 progeny had wiry, sterile phenotypes in both diploid and tetraploid lines. The sterility of the *spsgs3* null mutant was partially rescued, and fruits were obtained by grafting to

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wild-type (WT) stock and pollination with WT pollen. The resulting seeds contained the mutated alleles. Tomato yellow leaf curl virus proliferated at higher levels in *spsgs3* and *sprdr6* mutants than in the WT. Therefore, this protoplast regeneration technique should greatly facilitate tomato polyploidization and enable the use of CRISPR-Cas for *S. peruvianum* domestication and tomato breeding.

Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable crop, representing the sixth most economically important crop worldwide (http://www.fao.org/faostat/en/#data/QV). Wild tomato species are resistant to diverse biotic and abiotic stresses, and introgression lines are often used for tomato breeding (Schouten et al., 2019). *Solanum peruvianum* is considered the most variable tomato wild relative and represents an important gene pool for introducing early blight, leaf mold, fusarium wilt, Septoria leaf spot, root knot nematodes, bacterial canker, bud necrosis disease, and viruses resistance genes into modern tomato cultivars (Kaushal et al., 2020). The hallmarks of introgressions of resistance genes could be identified in the modern tomato genomes by functional characterization and whole-genome sequencing (WGS) analysis (Verlaan et al., 2013; Lin et al., 2014). *Solanum peruvianum* is a self-incompatible species and the existence of unilateral incompatibility has hitherto prevented its use as a female parent in the breeding (Hogenboom, 1972). De novo domestication of wild tomato was recently achieved within a short period by gene editing using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) (Li et al., 2018; Zsogon et al., 2018). Thus, CRISPR-Cas mutagenesis of wild tomato represents a strategy for tomato breeding and basic research.

Endopolyploidy, the occurrence of different ploidy levels within an organism, is widespread among plant taxa. It is often generated by endoreduplication where the complete genome is replicated without mitosis (Scholes and Paige, 2015). In tomato, cells with different ploidy are found in all organs (Smulders et al., 1994). Developmental stages such as young/old organs and growth conditions affect the ratio of ploidy level in cells. Furthermore, genome multiplication is a frequent occurrence during crop domestication. Many of the most economically important crops are polyploid, including potato (*Solanum tuberosum*), wheat (*Triticum aestivum*), and cotton (*Gossypium hirsutum*). Polyploidy conveys advantages in terms of genomic buffering, viability, and environmental robustness (Van de Peer et al., 2021). Triploids can also be used as seedless crops, such as watermelon (*Citrullus lanatus*) and bananas (*Musa acuminata*). Thus, CRISPR-Cas-edited tetraploid versions of crop species and their relatives represent important materials for crop breeding in the face of rapid climate change caused by global warming, among other challenges, as was recently demonstrated for tetraploid wild rice (*Oryza alta*) (Yu et al., 2021).

Therefore, it is important to establish a gene editing platform for polyploid crops and related species.

The CRISPR-Cas system uses *Agrobacterium tumefaciens*-mediated stable transformation to deliver DNA encoding Cas protein and single guide RNA (sgRNA) into the nuclei of tomato cells. As an alternative approach, CRISPR ribonucleoprotein (RNP) or plasmids harboring the Cas and sgRNA sequences can be introduced directly into protoplasts using transient transfection, allowing recombinant DNA-free plants to be regenerated to circumvent concerns about genetically modified organisms (GMOs) (Woo et al., 2015; Andersson et al., 2018; Lin et al., 2018; Hsu et al., 2019, 2021a, 2021b; De Bruyn et al., 2020; Yu et al., 2021). This protocol is important for use with hybrids or plants with a long juvenile period and for vegetative propagation because the transgenes from stable transformation (selection markers and CRISPR reagent genes) cannot be removed from these crops by crossing. Also, the progeny will be different from their heterozygous parental lines due to segregation. The gene editing efficiency and specificity could be validated by targeted sequencing (Woo et al., 2015; Nekrasov et al., 2017) or WGS (Fossi et al., 2019; Hsu et al., 2021b). Nevertheless, previous analysis paid little attention to the overall chromosomal changes, especially in polyploid regenerants (Fossi et al., 2019). WGS and copy number analysis would help to elucidate the potential genome instability after different steps of cell manipulations.

The protoplast regeneration gene editing system has two other major advantages: (1) Gene-edited transformants derived from tissue-culture-based *Agrobacterium*-mediated transformation are often chimeric, especially in eudicots (Shimatani et al., 2017). If the transformant is an edited/wild-type (WT) chimera and the edited allele occurs only in somatic cells (and not germ cells), edited alleles cannot be passed on to the next generation (Zheng et al., 2020). In protoplast regeneration, there is a low incidence of chimerism, and all mutated alleles detected in the T_0 generation can be transmitted to the next generation (Lin et al., 2018; Hsu et al., 2019, 2021a, 2021b). (2) The protoplast regeneration system can be used to introduce many CRISPR reagents and donor DNAs into plants for targeted insertion at the same time without the limitation of vector size (Hsu et al., 2019, 2021a). In addition, the second transfer step can be performed directly to obtain homozygous alleles in polyploids without self-fertilization which is very useful for hybrid, long juvenile period, and sterile plants (Hsu et al., 2019). However, the main bottleneck of this strategy is the difficulty of performing protoplast regeneration.

Here, we established a diploid/allotetraploid protoplast regeneration protocol for *S. peruvianum*, an important stress-resistant wild tomato, for use with CRISPR-Cas-mediated genome editing. We targeted several genes for editing, including *RNA-DEPENDENT RNA POLYMERASE6* (*SpRDR6*) and *SUPPRESSOR OF GENE SILENCING3* (*SpSGS3*), two key genes in the plant RNA silencing pathway ([Mourrain et al., 2000](#)) that mediate defense against tomato yellow leaf curl virus (TYLCV) ([Verlaan et al., 2013](#)); *PATHOGENESIS-RELATED PROTEIN-1* (*SpPR-1*) encoding the cysteine-rich secretory proteins antigen 5 and pathogenesis-related 1 protein (CAP)-derived peptide 1 (CAPE1) precursor ([Chen et al., 2014](#)) and *PROSYSTEMIN* (*SpProSys*), two pathogen-resistance peptide precursors; and *MILDEW RESISTANT LOCUS O* (*SpMlo1*) ([Nekrasov et al., 2017](#)). Targeting of these genes which was performed using two types of CRISPR reagents, plasmids and RNPs, yielded diploid and tetraploid transgene-free lines. Stable genome structures of ten plants, including one explant derived from stem cutting, three diploid regenerants and six tetraploid of *SpProSys* or *SpMlo1* RNP transfection regenerants were confirmed by WGS.

Results

Protoplast regeneration in *S. peruvianum*

To obtain a high proportion of tetraploid protoplasts, we analyzed the genome sizes of different explants (leaves and stems) using flow cytometry to determine the proportion of tetraploid cells. In leaves, the ratio of diploid to tetraploid nuclei was 5:1 ([Figure 1a](#)), and in stems, the ratio was 1:1 ([Figure 1b](#)). The same ratio was detected in protoplasts derived from stems ([Figure 1c](#)). Therefore, since stems had a higher proportion of tetraploid cells, we used them in subsequent studies to increase the proportion of tetraploid regenerated plants.

Using a method previously published for *Nicotiana benthamiana* ([Lin et al., 2018](#)), we successfully isolated *S. peruvianum* protoplasts from in vitro-grown shoots. We incubated the purified protoplasts in liquid medium consisting of half-strength Murashige and Skoog medium (1/2 MS), 0.4 M mannitol, 3% sucrose, 1 mg/L naphthalene-acetic acid (NAA), and 0.3 mg/L kinetin, pH 5.7, for 1 month in the dark, leading to the formation of fine, sand-like calli ([Figure 2a](#)). Next, we subcultured these calli in liquid medium containing 1/2 MS, 0.4 M mannitol, 3% sucrose, 2 mg/L kinetin, and 0.3 mg/L indole-3-acetic acid (IAA), pH 5.7, in the light ([Figure 2b](#)). After 1 month, these white calli turned green and were transferred to solid medium (1/2 MS, 0.2 M mannitol, 3% sucrose, and 2 mg/L kinetin; [Figure 2c](#)). We transferred the calli to fresh medium every month to induce the formation of small shoots ([Figure 2d](#)), which were incubated in medium without plant growth regulators until adventitious roots formed at the bottoms of the shoots ([Figure 2e](#)). Finally, we transferred the rooted plants to pots ([Figure 2f](#)) and grew them in the greenhouse ([Figure 2g](#)). The regenerated plants flowered ([Figure 2h](#)), fruited ([Figure 2i](#)), and produced seeds.

Optimized protoplast regeneration protocol

Compared with *N. benthamiana* ([Lin et al., 2018](#)), *S. peruvianum* protoplasts take longer to regenerate. According to our observations, the most important steps in the tomato regeneration process are those in liquid culture: callus induction in the dark (the first step) and callus proliferation in the light (the second step). Therefore, we tested several modifications to the composition of the culture medium to shorten the regeneration time. The results indicated that zeatin and 6-benzylaminopurine are the best hormonal treatments for the two liquid culture steps ([Supplemental Figure S1](#)), and zeatin is the best cytokinin for the third subculture step in solid medium ([Supplemental Figure S2](#)).

CRISPR-Cas9-targeted mutagenesis in *S. peruvianum*

We used this protoplast regeneration system to establish a method for CRISPR-Cas9-targeted gene mutagenesis of *S. peruvianum*. First, we used plasmids as CRISPR-Cas9 reagents for targeting mutagenesis of three important disease-resistance-related genes: *SpSGS3*, *SpRDR6*, and *SpPR-1*.
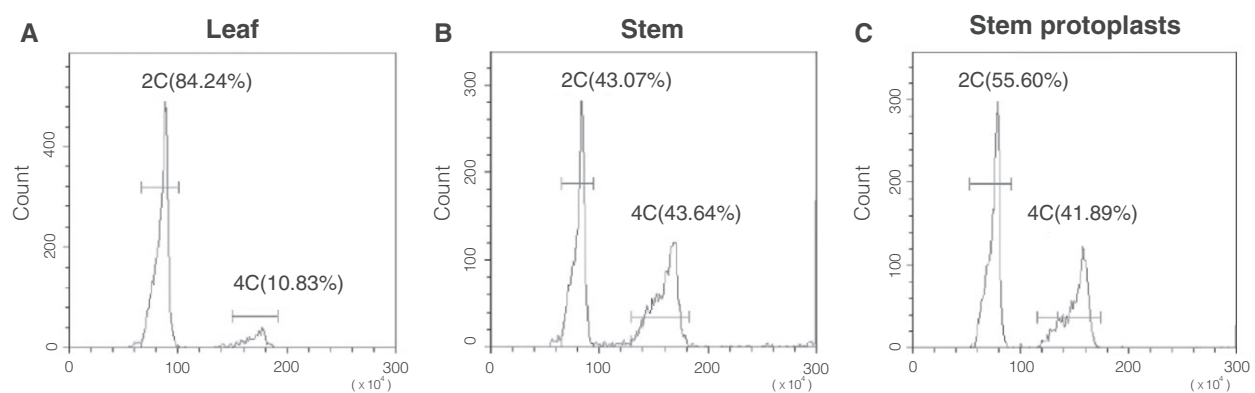
Figure 1 Flow cytometric analysis of the nuclear DNA contents of *S. peruvianum* tissues. The genome sizes of (a) leaves, (b) stems (c), and protoplasts derived from stems. X: fluorescence density; Y: count. Chicken erythrocyte nuclei (CEN: 2.5 Gb) were used as the calibration standard. The bar indicates the area used for counting nuclei. 2C: diploid; 4C: tetraploid. The number in brackets after the ploidy is the percentage of each different ploidy level versus the total counts.
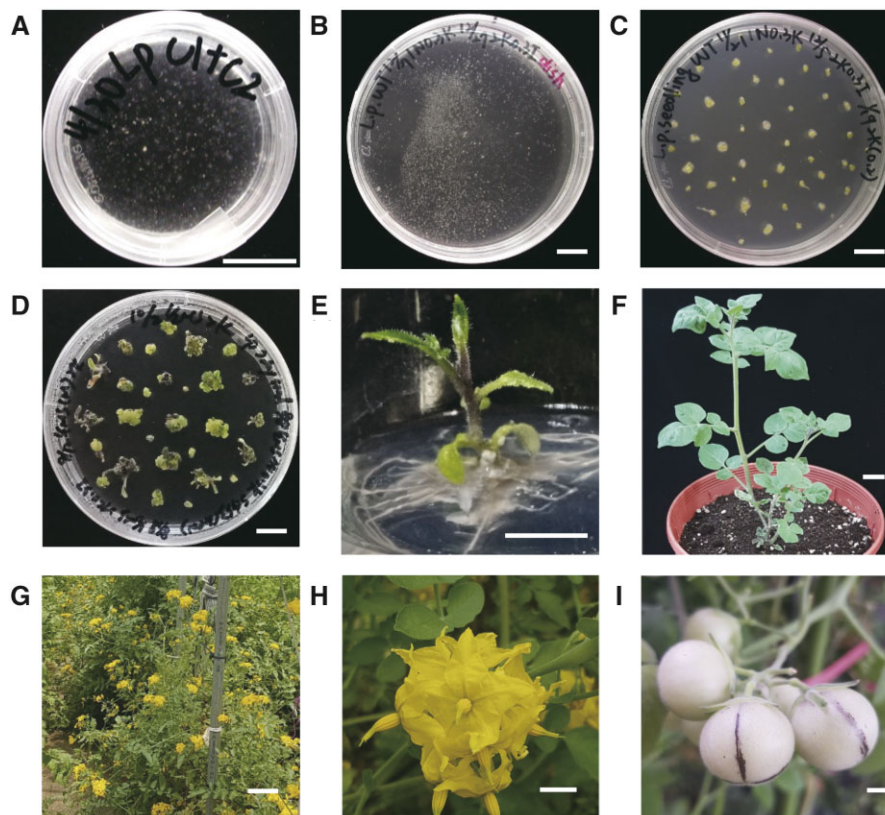
Figure 2 Regeneration of *S. peruvianum* protoplasts. a, Protoplasts incubated in 1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, 1 mg/L NAA, and 0.3 mg/L kinetin, pH 5.7 liquid medium for 1 month. b, Calli subcultured in 1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, 2 mg/L kinetin, and 0.3 mg/L IAA, pH 5.7 liquid medium in the light. c, Calli subcultured in 1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, 2 mg/L kinetin, pH 5.7, solid medium. d, Shoot bud formation after two subcultures in 2 mg/L kinetin solid medium. e, Adventitious root formation in plant growth regulator-free 1/2 MS solid medium supplemented with 3% sucrose. f, Regenerated plants after 1 month of growth in a pot. g, Regenerated plants grown in the field. h, Flowers of a regenerated plant. i, Fruits of a regenerated plant. Throughout, bars = 1 cm.

Table 1 CRISPR-Cas9 target sites and mutagenesis efficiencies

Reagent	Target gene	Target site		Mutation (%)
Plasmid	*SpSGS3*	ATTCCCCCCAGGATAAAAGC<u>GGG</u>	GCGCAATTGAATGGTTTACA<u>GGG</u>	8.3 (6/72)
		GTTCCTCCTGCTCTGAAGAA<u>TGG</u>	GTAACAATGCTGGATCAGGC<u>CGG</u>	
	SpRDR6	TTAAAGCTGGGACCATTGCG<u>AGG</u>	TGCGAGGTCGAATTGAAACA<u>CGG</u>	13.2 (5/38)
	SpPR-1	CCAGGAGAGAATCTTGCCAA<u>GGG</u>	CTCCGCCACCCACAATTCAG<u>AGG</u>	13.9 (10/72)
		GGGCTCGTTGCAACAACGGA<u>TGG</u>	TCTTGCAACTATGATCCTGT<u>AGG</u>	
		ACTATGATCCTGTAGGCAAT<u>TGG</u>	GATCCTGTAGGCAATTGGGT<u>CGG</u>	
		TGTCCGACCCAATTGCCTAC<u>AGG</u>		
RNP	*SpProSys*	TCATGGTGAAGTTTCACCTTT<u>TGG</u>	GGAGGATCACGCTTTGATGG<u>AGG</u>	45.8 (11/24)
	SpMlo1	GGTGTACCTGTGGTGGAGAC<u>TGG</u>	GTACAAAGTTAATCAAGAAT<u>AGG</u>	63.6 (14/22)

Note: Underlined letters indicate the protoplast adjacent motif sequence.

In the *SpSGS3* experiment, we chose four target sites (Table 1), and the total efficiency of mutagenesis was 8.3%. Based on sequencing results, mutations occurred in all three target sites except GTAACAATGCTGGATCAGGC. Among these, GCGCAATTGAATGGTTTACA was targeted the most effectively, and mutations at this position were observed in all mutants (Supplemental Table S1). spsgs3#6 (2n), #11 (2n), and #13 (2n) are the null mutants and spsgs3#6 contains four mutated alleles. *SpSGS3#7* (4n) also contains three mutated alleles and one non-mutated WT allele. A 68-bp insertion from the vector was detected in spsgs3#11 (2n).

In the *SpRDR6* experiment, we selected two target sites (Table 1). Based on the sequencing results, both target sites could be mutated by CRISPR-Cas9, with a total mutation efficiency of 13.2%. TTAAAGCTGGGACCATTGCG gave the best results, as all five mutant plants contained mutations at this target site. The mutation TGCGAGGTCGAATTGAAACA was only identified in *SpRDR6#38* (Supplemental Table S2). All regenerated mutants were heterozygous, and *SpRDR6#38* had two mutated alleles and at least one WT allele.

In the *SpPR-1* experiment, seven target sites were selected and used to construct two vectors. These two constructs,

harboring sgRNAs targeting seven target sites, were co-transfected into protoplasts (Table 1). Among the 10 regenerated mutants, 4 contained fragment deletions, indicating that at least two cleavages had occurred. Except for TGTCCGATCCAGTTGCCTAC and CTATGATCCTGTAGGCAAC there were no mutations in the target sites; the five other sgRNAs caused mutations at the expected positions. The mature CAPE1 peptide is derived from the C-terminal end of tomato PR-1b. sppr-1#28, #31, and #52 were mutated only in the target sites located in CAPE1, all at ATCCTGTAGGCAACTGGAT, resulting in a 5-bp deletion. All SpPR-1 mutants were null mutants except for SpPR-1#72 (Supplemental Table S3).

In the experiments with N. tabacum (Lin et al., 2018) and SpSGS3 (Supplemental Table S1), the use of plasmid CRISPR reagent may still result in foreign DNA insertions. Therefore, RNP is used as a CRISPR reagent to achieve DNA-free gene editing. Here, we delivered two RNPs that target sites located in SpProSys to protoplasts and regenerated the transfected protoplasts into plants. Upon sequencing of the 24 regenerated SpProSys plants, 11 showed target mutagenesis (45.8%, Table 1). Prosystemin is a precursor of systemin, which is processed by phytaspase (Beloshistov et al., 2018). The target site GGAGGATCACGCTTTGATGG is at the C terminus of SpProSys, which is the position of systemin, and the mutations in lines #5, #16, and #19 occurred only at this site (Supplemental Table S4). Using two published SlMlo1 target sites (Nekrasov et al., 2017), we synthesized RNPs targeted to these sites in vitro and simultaneously delivered them into protoplasts. Of the regenerated calli and plants, 63.6% showed targeted mutagenesis (Table 1 and Supplemental Table S5).

Analysis of the genome sizes, phenotypes, and progeny of diploids and tetraploids

A higher proportion of tetraploid cells was observed in protoplasts derived from diploid stems compared to leaf tissue (Figure 1). In addition, during target gene genotyping, we observed that some mutants contained more than three alleles. For example, SpRDR6#38 contained three alleles ($+1$bp, -7bp, and WT, Supplemental Table S2), and its genome size was 4.40 ± 0.03 pg. Therefore, targeted mutant plants of tetraploids can be obtained using this method. We performed karyotype analysis of these regenerated plants (T_0, sterile mutants) or their offspring (T_1) to confirm the chromosome numbers (Figure 3). Except for a SpPR-1 tetraploid without targeting regenerant, we obtained diploid and tetraploid regenerated plants with or without targeting mutations derived from plasmid CRISR-Cas9 reagent-transfected protoplasts (Figure 3 and Supplemental Table S6). Similar results were obtained for SpProSys RNP transfection (Supplemental Figure S3 and Supplemental Table S7). The ploidy of the plants that were regenerated from transfected protoplasts is provided in Supplemental Tables S6 and S7. These results indicate that most tetraploid plants were

derived from tetraploid protoplasts from the explants rather than by protoplast fusion caused by the presence of PEG-Ca^{2+} in the transfection medium.

In regenerated plants derived from SpSGS3 transfection, the tetraploids had a reduced seed set (Supplemental Figure S4a). The seeds of tetraploids were larger than those of diploids; this phenomenon was also observed in tetraploid regenerated plants derived from transfection with other CRISPR reagents (Supplemental Figure S4b). The tetraploid plants grew more slowly than the diploid plants (Supplemental Figure S4c). The leaf edges of tetraploid plants were more rounded than those of the diploid plants (Supplemental Figure S4c).

We subjected the offspring of SpSGS3#7 and #10 (Supplemental Figure S5); SpRDR6#6, #33, and #38 (Supplemental Figure S6); and sppr-1#52 and #61 (Supplemental Figure S7) to target gene sequencing. Except for sppr-1#52, which contained one mutant locus not present in the parent, all other offspring had the same mutated locus as the parent. These results demonstrate that these mutated loci can be transmitted to the next generation in diploids and tetraploids.

Stable genome structures in diploids and tetraploids

To further confirm the stability of genome structure in regenerants, we performed WGS of ten samples, including one diploid plant propagated by stem cutting (SpB), three diploids and six tetraploids derived from SpProSys or SpMlo1 RNP transfection (Supplemental Table S7). Taking into account the different genome sizes between diploid and tetraploid plants, each sample was sequenced to the anticipated 30x genome coverage. That is, 141–171 million pair-end reads were sequenced for diploid plants and at least 252–373 million pair-end reads were sequenced for tetraploid plants (Supplemental Table S7).

Multiple analysis strategies were used to study the genome structures. Despite the low mapping rate of both diploid and tetraploid samples at some chromosome locations, sequencing coverage analysis did not show inconsistent coverage changes between samples (Supplemental Figure S8). Deletion of large chromosomal segments, which were commonly seen in aneuploid cells (Musacchio and Salmon, 2007) cause allelic imbalance. By calculating heterozygous allele frequency of sequenced plants, we did not identify abnormal allele frequency variations or loss of heterozygosity (Figure 4a). A Bayesian approach to determine copy number variations (CNVs) along chromosomes and compared between uneven sequencing depth of samples did not identify abnormal copy number changes in sequenced plants (Figure 4b). Taking these findings together, we concluded that there is no abnormal chromosomal gain or loss in diploid and tetraploid plants. Neither the protoplast regeneration process nor the CRISPR reagents caused detectible chromosomal changes.
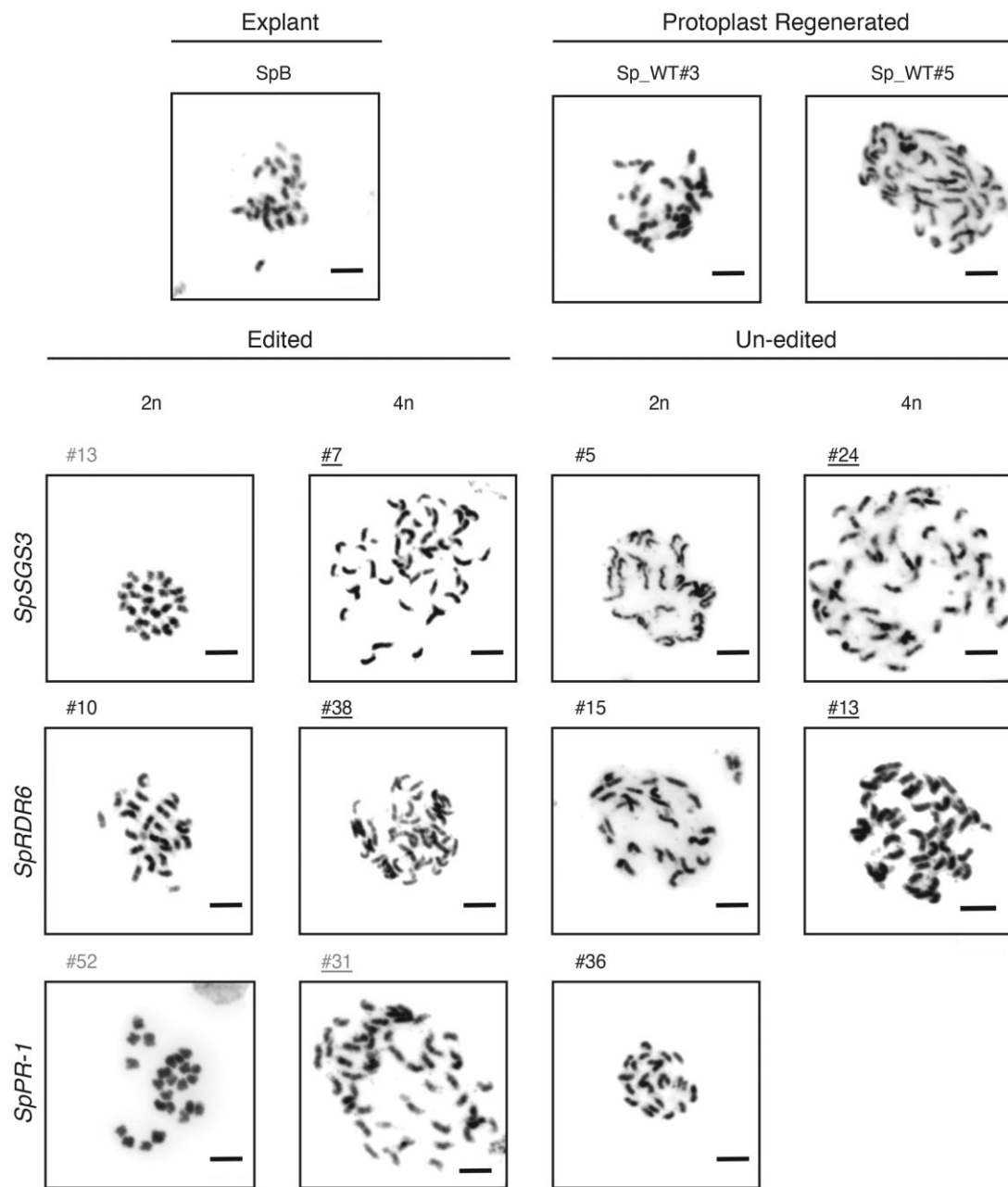
Figure 3 Karyotypes of *S. peruvianum* plants regenerated from protoplasts. Gray font: null mutant. Black font: heterozygous or WT. Underline: *4n*. Bars = 5 μm.

### *spsgs3* and *sprdr6* diploid and tetraploid null mutants show wiry phenotypes

The regenerated plants containing a WT allele(s) produced flowers and fruits (Figure 2) with morphology and development similar to those of WT plants in the greenhouse. Biallelic *spsgs3* mutants (carrying two distinct genome-edited alleles: *spsgs3#11*, Figure 5; *spsgs3-6* and *spsgs3-13*; Supplemental Figure S9a) had a wiry leaf phenotype and abnormal flowers, which is similar to the previously reported *sgs3* domesticated tomato mutants (Yifhar et al., 2012).

Among the six progeny of *SpSGS3#7*, two progeny harbored the mutated alleles only (Supplemental Figure S5); these plants also showed a wiry phenotype (*spsgs3#7-2*;

Supplemental Figure S9b). A similar phenomenon was also observed in the *SpRDR6* regenerants. Although all *SpRDR6* T$_0$ plants were heterozygous and contained WT *SpRDR6* alleles in their genomes, no wiry phenotypes were observed. The *SpRDR6#33* and *SpRDR6#38* offspring had wiry phenotypes (*sprdr6#33-G*, Figure 5b; *sprdr6#38-16*, Supplemental Figure S9b). The pollen of both null T$_0$ and T$_1$ mutated plants, including *SpSGS3* and *SpRDR6* mutants, was abnormal (Supplemental Figure S9c) and failed to produce seeds.

Because *AUXIN RESPONSE FACTOR3* (*ARF3*) and *ARF4* are the target genes of trans-acting secondary siRNA3 (*TAS3*), whose biogenesis requires RDR6 (Marin et al., 2010), we investigated the transcript levels of these genes in WT, *spsgs3*
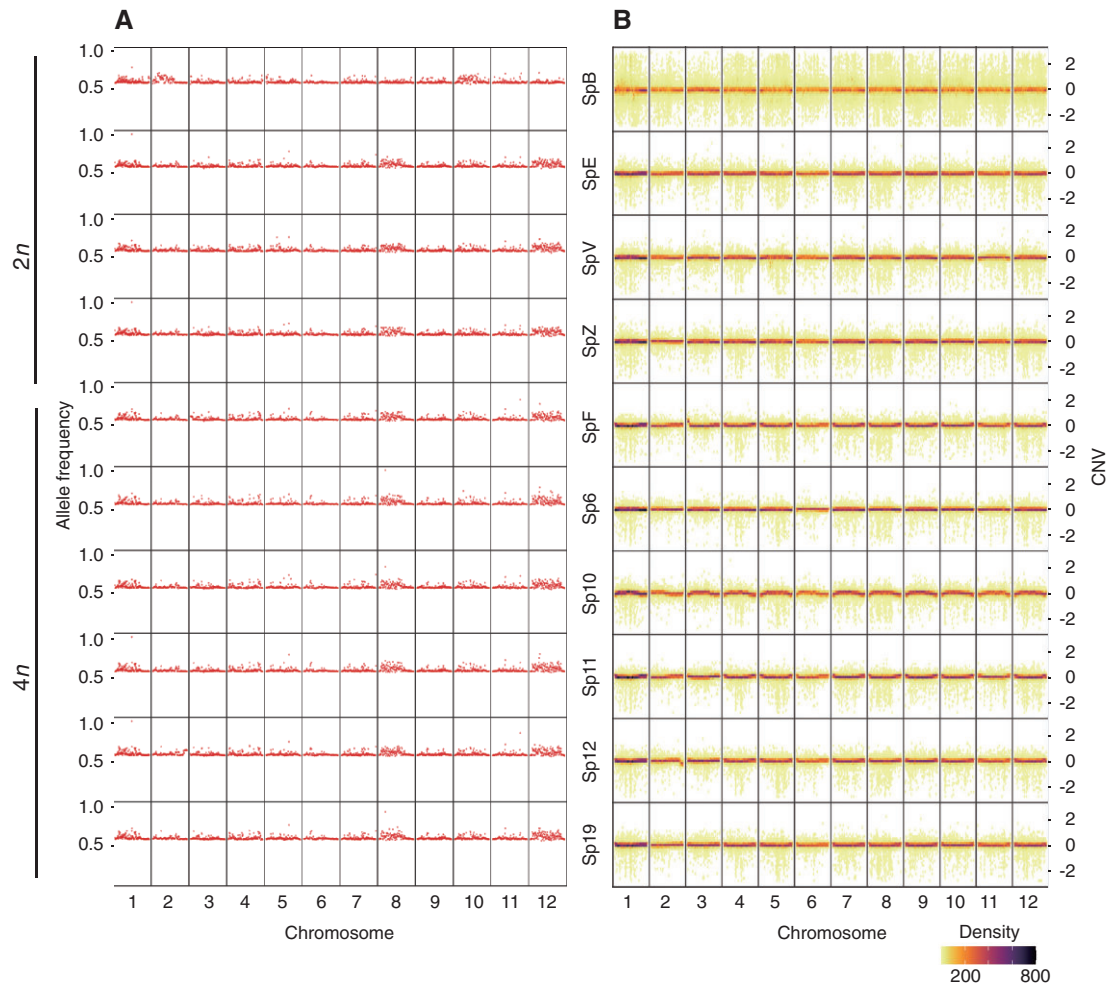
Figure 4 Stable genome structures in plants regenerated from stem cutting and protoplasts. a, Heterozygous allele frequency of WGS samples. The heterozygous allele frequency was attained by dividing the read depth of the heterozygous allele (labeled as 0/1 by GLnexus) by the total read depth of the variant. Heterozygous frequency is plotted using 10-kb chromosome window size on the X axis. A value of heterozygous allele frequency 0.5 indicates the frequency of the heterozygous genotype (0/1) from the DeepVariant is 0.5, regardless the ploidy level. b, CNV of WGS samples. CNV was predicted as 3 kb fragment size with minimum 10 fragments. Predicted CNV is plotted using 30 bins per chromosome on the X axis. Dot colors indicate the CNV density per bin. A value of zero on the Y axis indicates no copy number change was detected. Values above zero indicate copy number gain and below zero indicate copy number loss.

and $sprdr6$ plants (Figure 5a). The $spsgs3$ null mutants (T_0: $spsgs3$#11 and $spsgs3$#13; T_1: $spsgs3$#7-1) lacked $SpSGS3$ expression. In contrast to the WT, the transcript levels of $SpARF3$ and $SpARF4$ were increased in the $spsgs$ mutants, not only for null diploid mutants $spsgs3$#11 and $spsgs3$#13 but also for tetraploid mutant $spsgs3$#7-1 (Figure 5a). Similarly, the transcript levels of $SpARF3$ and $SpARF4$ were also increased in the $SpRDR6$ T_1 mutant $sprdr6$#33-G (Figure 5a).

TYLCV proliferation

We evaluated the infectivity of TYLCV in the mutants by in vitro inoculation (Al Abdallat et al., 2010). After 8 weeks in vitro inoculation, plant growth was severely retarded (Supplemental Figure S10) and leaf morphology changed in the T_1 diploid $spsgs3$#11 (Figure 6a) and the T_2 tetraploid $sprdr6$#38-6 (Figure 6b). Compared to the WT, all of the

null mutants ($spsgs3$/$sprdr6$ and diploid/tetraploid) showed higher levels of TYLCV accumulation (Figure 6, a and b, Supplemental Figure S10).

Grafting rescued the fertility of the sgs3#11 null mutant

We used WT pollen for hybridization, which failed to pollinate the fruits of the $spsgs3$ and $sprdr6$ null mutants. Based on these results, these mutants could not produce the substrate(s) needed for the development of male or female reproductive organs. However, using grafting, the substrate(s) produced in WT stock was successfully transported to the $spsgs3$#11 scion (Figure 7a). Although there were no substantial differences in leaf (Figure 7b) or flower morphology (Figure 7c), $spsgs3$#11 failed to produce viable pollen (Supplemental Figure S9c) and the pollen viability of $spsgs3$#11 increased to 20% by grafting to the WT stock.
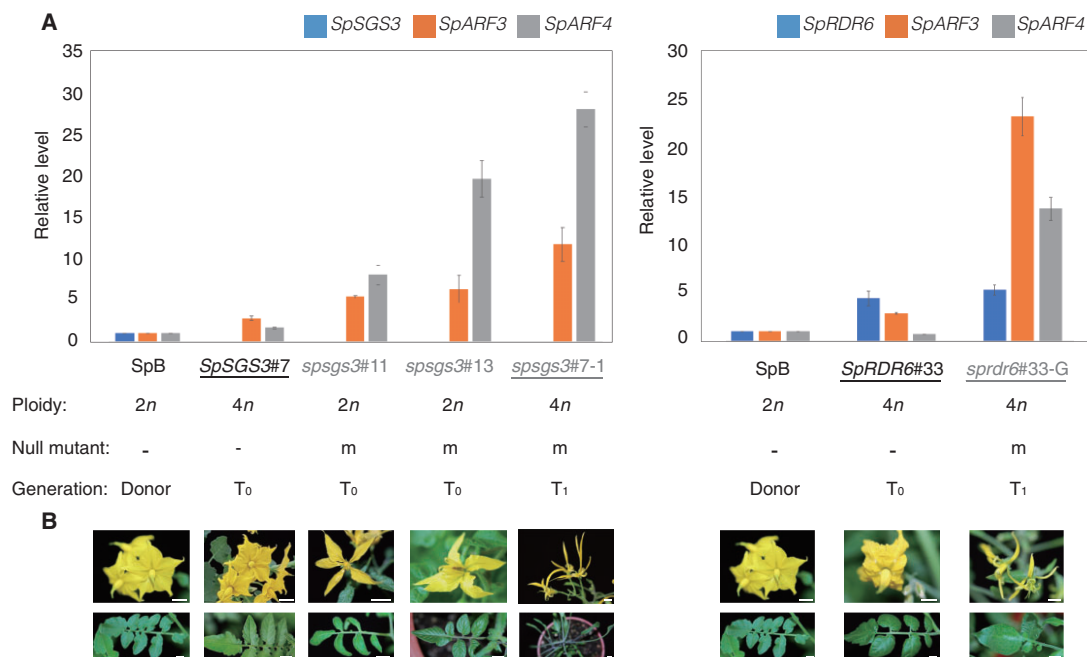
Figure 5 Gene expression and phenotypic profiles of *S. peruvianum* sgs3 and rdr6 mutants. a, RT-qPCR analysis of auxin response regulator genes (SpSGS3, SpARF3, SpARF4, and SpRDR6) in the WT and protoplast-derived regenerants ($n = 3$). T_0: regenerated plants derived from protoplasts. T_1: seedlings derived from T_0 plants. Error bars represent the stand deviation. b, Phenotypes of *spsgs3* and *sprdr6* mutants. Bars = 1 cm.
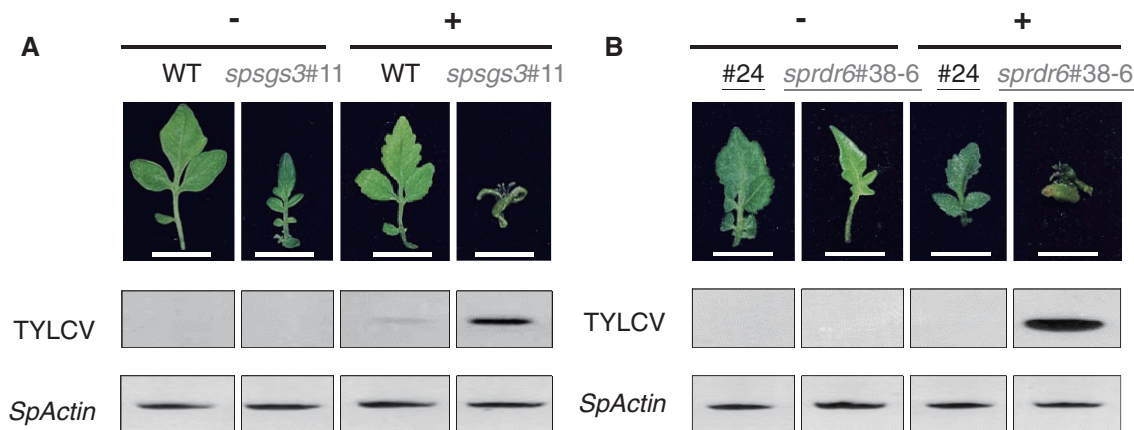
Figure 6 Symptoms and TYLCV proliferation on in vitro-cultured *S. peruvianum* plants inoculated with the infectious TYLCV clone. a, Diploid WT and spsgs3#11 mutant. b, Tetraploid regenerant (#24) and *sprdr6#38-6* mutant. TYLCV: PCR product of the TYLCV fragment. *SpActin*: PCR product of *SpActin*. Gray: null mutant. Black: Un-edited tetraploid regenerated plant (#24) or the WT. Underline: 4n. Bars = 1 cm.

Grafted *spsgs3#11* produced fruits (Figure 7d), but non-grafted *spsgs3#11* did not. The fruits from *spsgs3#11* scions were smaller (Figure 7e) and contained fewer seeds than the WT (Figure 7f). Genotyping indicated that all of the progeny harbored *spsgs3#11* mutated alleles (Figure 7g).

Discussion

It remains challenging to introduce desired alleles from multiple wild species into a single cultivar. In the history of the development of tomato disease resistant cultivars, the root knot nematode resistance gene *Mi-1* was introduced from *S. peruvianum* and the TYLCV resistance gene *Ty-1 1* was introduced from *Solanum chilense*. These two introgressed fragments are located at a nearby genomic location (26.6–27.7 Mb versus 30.9–32.5 Mb) on chromosome 6 and remain intact under severe recombination suppression (Lin et al., 2014). It was difficult to combine both genes into a single cultivar likely due to chromosomal inversions or close to the centromeric location. Development of CRISPR-Cas editing in wild tomatoes would allow the introduction of the desired alleles from wild species without the limitation of recombination frequency or the cost of potential linkage drag.

In addition to its use in wild tomatoes, CRISPR is also utilized in commercial varieties of *S. lycopersicum*. Dozens of studies using this technique in tomato have been published, most involving breeding trials for traits such as quality (fruit
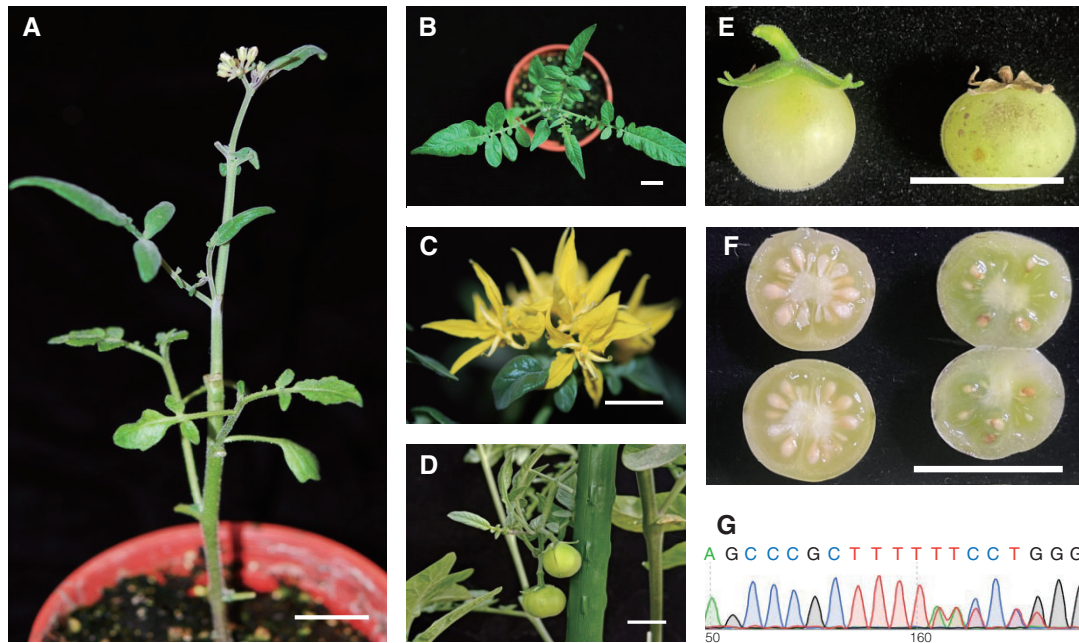
Figure 7 Growth of a sterile *spsgs3* #11 plant grafted with WT stock. a, Grafted plant. Leaves (b), flowers (c), and fruit (d) of spsgs3 #11 scion. Mature fruit (e) and seeds (f) of WT stock (left) and *spsgs3* #11 scion (right). g, Results of Sanger sequencing of the seedling derived from *spsgs3* #11 scion fruit, which is heterozygous, harboring *spsgs3*#11 mutated alleles mixed with the WT allele. Bars = 1 cm.

architecture, color, metabolism, and postharvest), anti-stress (biotic and abiotic stress), and domestication (Li et al., 2018; Zsogon et al., 2018). These studies were performed using several CRISPR platforms established in tomato, including (1) Cas9 (Brooks et al., 2014) and Cas12a (Bernabe-Orts et al., 2019), to generate DNA double-strand breaks that are preferentially repaired by non-homologous end joining to introduce target mutations; (2) precise modification of plant genomes using DNA repair templates via homologous recombination (Cermak et al., 2015); and (3) the cytidine base editor, an inactive Cas9 fusion with cytidine deaminase, which converts cytosine to uracil without cutting DNA and introducing mutations (Shimatani et al., 2017). Therefore, CRISPR is emerging as a powerful tool for tomato breeding.

For commercial breeding, however, it is desirable to produce DNA-free plants to avoid concerns about GMOs. Although there are several reports demonstrating successful DNA-free genome editing via biolistic methods in many crops (Svitashev et al., 2016; Liang et al., 2018; Banakar et al., 2020), protoplast regeneration systems have higher efficiency. Many studies have been performed using RNPs and plasmids to achieve DNA-free genome editing (Woo et al., 2015; Andersson et al., 2018; Lin et al., 2018; Hsu et al., 2019, 2021a, 2021b; De Bruyn et al., 2020; Yu et al., 2021). These reports indicate that it is possible to establish protoplast regeneration platforms for tomato and various target crops/plants.

Tomato and related species have been important materials in the development of protoplast isolation and regeneration techniques. Tomato protoplasts were isolated by enzymatic digestion, and this landmark achievement allowed

sufficient amounts of protoplasts to be obtained for further application (Cocking, 1960). *Solanum peruvianum* was the first tomato-related species for which a protoplast regeneration system was reported, and such systems have subsequently been achieved in many tomato and wild tomato species (Kut and Evans, 1982). In this study, we combined these techniques to achieve DNA-free genome editing of a wild tomato. This method could be applied to other tomato-related species to facilitate breeding.

Although protoplast regeneration was first reported 50 years ago (Takebe et al., 1971), it still represents a major bottleneck in DNA-free genome editing. The shoot regeneration capacity in *Lycopersicon* is highly genotype dependent (Peres et al., 2001). Understanding how a cell is regenerated into a complete plant is an important topic of scientific and agricultural research (Sugimoto et al., 2019), but information about this process is still limited. Such knowledge could be applied to develop efficient tissue culture, gene transformation, and genome editing systems, tools that are important for de novo plant domestication (Li et al., 2018; Zsogon et al., 2018; Maher et al., 2020; Yu et al., 2021). In this study, we assessed the effects of plant growth regulators in the medium on protoplast regeneration. In addition to the chemical approach, several genes encoding morphogenic regulators have been identified and used to improve the efficiency of plant regeneration. It is possible to control the expression of these genes to establish a non-tissue-culture regeneration system for gene editing (Maher et al., 2020).

In addition to their roles in the domestication of wild species, polyploid crops have other benefits, including larger plants (Chung et al., 2017), and higher yields (Chen et al.,

2018). In addition, triploid crop cultivars of species such as bananas and watermelons can produce commercially desirable seedless fruits. Most previous methods for chromosome multiplication have used colchicine. This procedure is complicated and inefficient, producing regenerated plants with mixed cell populations of various ploidy levels (Cola et al., 2014). Similar to haploid culture, in this report, using isolated protoplasts from polyploid cells in explants for regeneration and gene editing, we were able to obtain edited polyploid regenerated wild tomatoes without colchicine treatment. This phenomenon has also been reported in other plant species. In witloof chicory plants (*Cichorium intybus* var. *foliosum*) generated from CRISPR/Cas-edited protoplasts, 77.2% diploid and 21.5% tetraploid plants were produced and the remaining 1.3% consisted of haploids, hexaploids, and mixoploids (De Bruyn et al., 2020). Therefore, explants containing high proportions of polyploidized cells could be widely used for protoplast regeneration for crop polyploidization. However, in this study, we found no substantial enlargement in the leaves or flowers of tetraploid versus diploid lines, similar to the pattern reported for tetraploid tomatoes (Nilsson, 1950).

In addition to technological difficulties, the presumed mutagenicity of protoplast regeneration is another reason why researchers are reluctant to use this system as a gene editing platform. Indeed, WGS has revealed widespread genome instability in potatoes regenerated from protoplasts (Fossi et al., 2019), which has increased the concerns about this technology. The original purpose of protoplast regeneration was to use protoplast fusion to improve hybridization or as a platform for mutagenesis. Since only successful cases of mutation or fusion have been reported, and most such experiments have not been compared with other tissue culture methods, many researchers have the impression that protoplast regeneration readily leads to mutagenesis. In fact, other tissue culture technologies, including multiple shoot proliferation (Lin et al., 2007) and somatic embryogenesis (Lin et al., 2007), can also cause mutations. Although this study involved the use of PEG-Ca^{2+} in the transfection process, which could promote cell fusion, non-transfected tetraploid regenerated plants were also obtained. Based on our finding that the proportion of tetraploid regenerated plants was similar to that of shoot explants, we believe that the formation of polyploid regenerated plants was primarily due to the presence of polyploid cells in the explants. In addition to protoplast regeneration, there are also opportunities to obtain polyploid plants using other tissue culture technologies (Chung et al., 2017). In an *Agrobacterium*-mediated transformation experiment in tomato, the rate of tetraploid transgenic plants ranged from 24.5% to 80% and depended on both the genotype and the transformation procedure (Ellul et al., 2003). In *Arabidopsis thaliana* T-DNA insertion mutagenesis, large-scale genomic rearrangements have occurred (Pucker et al., 2021). Therefore, we believe that protoplast regeneration is an excellent tool for gene editing as well as other transgenic platforms.

Unlike the previous report of widespread genome changes in the autotetraploid potato (Fossi et al., 2019), the WGS analysis in this study does not identify aneuploidy and abnormal chromosomal changes in either diploid or tetraploid regenerants. Chromosomes in the autotetraploid genome, such as cultivated potato, were derived from the merging of two different chromosome sets (Van de Peer et al., 2021). On the other hand, tetraploid plants in this study, which were derived from chromosome doubling, contained the two identical sets of chromosomes. As the tissue culture steps caused a certain level of cell stresses, pairing of nonhomologous chromosomes in the autotetraploid genomes (Fossi et al., 2019) likely has a higher probability of incorrect chromosome pairing than in the allotetraploid genomes (Hsu et al., 2019; Yu et al., 2021). Incorrect chromosome segregation during mitosis in the autotetraploid cells likely has a higher probability of evading the spindle-assembly checkpoint (Musacchio and Salmon, 2007). Furthermore, by analyzing changes in the allele frequency and CNV, we confirmed that the CRISPR-Cas9 editing did not introduce large scale chromosomal changes and unintended genome editing sites (Hsu et al., 2021b).

In this study, all tetraploid and diploid *spsgs3* and *sprdr6* null mutants had wiry phenotypes, similar to other microRNA biogenesis null mutants in tomatoes (Yifhar et al., 2012; Brooks et al., 2014). *sgs3* and *rdr6* null mutants show various phenotypes in different species. *N. benthamiana spsgs3* and *sprdr6* mutants have a wiry flower morphology and sterile phenotype, but their leaves are similar to those of the WT (Hsu et al., 2021a). The *Arabidopsis sgs3* mutant shows no substantial phenotype (Adenot et al., 2006). Therefore, we would like to discover ways to improve the fertility of these mutants.

Grafting is a traditional agricultural tool that is used to control flowering, improve fruit quality, and increase resistance to biotic and abiotic stress (Haroldsen et al., 2012). In *N. benthamiana*, gene silencing was transmitted with 100% efficiency in a unidirectional manner from silenced stocks to nonsilenced scions expressing the corresponding transgene (Palauqui et al., 1997). In this study, a mutant of *SpSGS3*, an RNA silencing-related gene, was used as a scion and grafted onto RNA-silenced normal WT rootstock. The fertility of *spsgs3#11* scions was rescued, and they produced seeds with mutated alleles. In *Arabidopsis*, more than 3,000 mobile genes have been identified. The mRNA from these genes could be transported long distance, including *SGS3* mRNA (Thieme et al., 2015). In addition to mRNA, organellar DNA, proteins, and plant growth regulators can also move across graft unions (Haroldsen et al., 2012). Whether these mobile substances were also involved in rescuing the fertility of the *spsgs3#11* scions or whether grafting with WT plants could rescue other sterile mutants of mobile RNA requires further investigation.

Conclusions

To obtain tetraploid *S. peruvianum* DNA-free genome-edited plants, we used in vitro-grown shoots, which contain high

proportions of tetraploid cells, as explants for protoplast isolation and regeneration. The medium components were optimized, and genome-edited regenerants were obtained within 6 months. This is the first study in *S. peruvianum* describing the use of both RNP and plasmid CRISPR reagents for DNA-free genome editing, yielding a targeted mutagenesis efficiency of 60% without the need for marker gene selection. Diploid and tetraploid heritable mutants were obtained for all pathogen-related genes targeted in this study, including *SpSGS3*, *SpRDR6*, *SpPR-1*, *SpProSys*, and *SpMlo1*, and the expected phenotypes were obtained. In comparative WGS analysis, protoplast derived CRISPR-Cas9 edited plants, either diploid or tetraploid, showed stable genome structure. The proliferation of TYLCV, an important viral disease of tomato, was increased in *spsgs3* and *sprdr6* null mutants. The reproductive growth defect of the *SpSGS3* mutant was successfully rescued by grafting with WT stock. The protocols and materials described in this study will be useful for tomato breeding.

Materials and methods

Plant materials
Sterile *S. peruvianum* plantlets (seeds obtained from the National Plant Genetic Resources Center, Taiwan Agricultural Research Institute with US National Plant Germplasm System Accession number: PI 126441, https://npgsweb.ars-grin.gov/gringlobal/accessiondetail?id=1133100) were propagated by cutting and growing them in 0.5 MS medium supplemented with 30 mg/L sucrose and 1% (v/v) agar, pH 5.7. The plantlets were incubated in a 26°C culture room (12 h light/12 h in dark, light intensity of 75 μmol m^{-2} s^{-1}). The plantlets were cut and subcultured in fresh medium monthly.

Protoplast isolation and transfection
Protoplast isolation and transfection of *S. peruvianum* were performed following our previously published method with minor modifications (Hsu et al., 2019). Protoplasts were isolated from the stems and petioles of in vitro-grown plantlets. Five or more stems (~5 cm/each, total 0.2–0.25 g) were used to isolate roughly 1×10^6 protoplasts. These materials were place in a 6-cm glass Petri dish with 10 mL digestion solution (1/4 MS liquid medium containing 1% [v/v] cellulose and 0.5% [v/v] macerozyme, 3% [v/v] sucrose, and 0.4 M mannitol, pH 5.7) and cut into 0.5-cm-wide strips longitudinally. The material was incubated at room temperature in the dark overnight. The digested solution was diluted in 10 mL W5 (154 mM NaCl, 125 mM CaCl$_2$, 5 mM KCl, 2 mM MES, and 5 mM glucose) solution and filtered through a 40-μm nylon mesh. The sample was centrifuged at low speed (360× g) for 3 min to collect the protoplasts. The protoplasts were purified in 20% (v/v) sucrose solution and washed three times with W5 solution. The protoplasts were transferred to transfection buffer (1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, 1 mg/L

NAA, and 0.3 mg/L kinetin, pH 5.7) and adjusted to a concentration of 3×10^5 cells/mL.

The protoplasts were transfected with plasmids by PEG-Ca^{2+}-mediated transfection (Woo et al., 2015; Lin et al., 2018). A 400-μL sample (1.2×10^5 protoplasts) was combined with 40 μL of CRISPR reagent (DNA: 20–40 μg; RNP: 10 μg) and mixed carefully. The same volume (440 μL) of PEG-Ca^{2+} solution was added to the sample, mixed, and incubated for 30 min. To end the reaction, 3 mL of W5 was added and the sample was mixed well. Transfected protoplasts were collected by centrifugation at 360× g for 3 min. The protoplasts were washed with 3 mL of W5 and centrifuged at 360× g for 3 min. The target sites are shown in Table 1.

CRISPR/Cas reagents
The SpCas9 vector for dicot transformation (pYLCRISPR/Cas9P35S-N) (Ma et al., 2015) was isolated using a Plasmid Midi-prep kit (Bio-Genesis). Preparation of Cas9 protein and sgRNA and Cas9 RNP nucleofection were performed according to Huang et al. (2020). Cas9 RNP complexes were assembled immediately before nucleofection by mixing equal volumes of 40 μM Cas9 protein and 88.3 μM sgRNA at a molar ratio of 1:2.2 and incubating at 37°C for 10 min.

Protoplast regeneration
Pooled protoplast DNA was used as a template to amplify the target genes for validation by sequencing. The putatively edited protoplasts were transferred to 5-cm-diameter Petri dishes containing 3 mL 1/2 MS liquid medium supplemented with 3% (v/v) sucrose, 0.4 M mannitol, 1 mg/L NAA, and 0.3 mg/L kinetin for plant regeneration. Calli formed from the protoplasts after 1 month of incubation in the dark. The calli were subcultured in a 9-cm-diameter Petri dish containing fresh medium with cytokinin for 3–4 weeks in the light. Calli that had turned green were transferred to solid medium containing the same plant growth regulators. The explants were subcultured every 4 weeks until shoots formed after several subcultures. The shoots were subcultured in solid rooting medium (HB1: 3 g/L Hyponex No. 1, 2 g/L tryptone, 20 g/L sucrose, 1 g/L activated charcoal, and 10 g/L Agar, pH 5.2) for adventitious roots formation.

Analysis of the genotypes of regenerated plants
Two pairs of primers were designed to amplify the sgRNA-targeted DNA region for each target gene. The PCR conditions were 94°C for 5 min, 35 cycles of denaturing (94°C for 30 s), annealing (55°C for 30 s), and polymerization (72°C for 30 s), followed by an extension reaction at 72°C for 3 min. The PCR product was sequenced by Sanger sequencing to confirm mutagenesis. The multiple sequences derived from mutated regenerated plants were bioinformatically separated using Poly Peak Parser (http://yosttools.genetics.utah.edu/PolyPeakParser/; (Hill et al., 2014)) or further confirmed by sequential T/A cloning and sequencing. The primer sequences are listed in Supplemental Table S8.

Estimation of genome size

Fresh leaves were finely chopped with a new razor blade in 250 μL isolation buffer (200 mM Tris, 4 mM MgCl$_2$-6H$_2$O, and 0.5% Triton X-100) and mixed well (Dolezel et al., 2007). The mixture was filtered through a 40-μm nylon mesh, and the filtered suspensions were incubated with a DNA fluorochrome (50 μg/mL propidium iodide containing RNase A). The samples were analyzed using a MoFlo XDP Cell Sorter (Beckman Coulter Life Sciences) and an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Chicken erythrocyte (BioSure) was used as an internal reference.

WGS

Leaves of *S. peruvianum* regenerates were harvested and genomic DNA was extracted using two independent protocols. A nuclei isolation protocol (Sikorskaite et al., 2013) was used on the WT (SpB) sample to recover higher quality and quantity of DNA samples. Briefly, nuclei were extracted by 36 mM sodium bisulfite, 0.35 M Sorbitol, 0.1 M Tris-base, 5 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, and 2 mL 5% (v/v) *N*-lauroylsarcosine sodium salt. The genomic DNA was then extracted by chloroform–isoamyl alcohol (24:1), ethanol precipitation, and further cleaned up by DNeasy Blood & Tissue Kit (69504, Qiagen) and AMPure (Beckman Coulter Life Sciences). The other nine samples used the chloroform–isoamyl alcohol (24:1) for DNA extraction, followed with Zymo Genomic DNA Clean & Concentrator-25 (D4064, Zymo), and Zymo OneStep PCR Inhibitor Removal Kit (D6030, Zymo) to obtain high quality genomic DNA. DNA integrity was checked using the D1000 Screen Tape on the Agilent TapeStation 4150 System with DIN value >8. Genomic DNA were sheared using a Covaris E220 sonicator (Covaris) and paired-end sequencing libraries were constructed by the NEBNext Ultra DNA Library Prep Kit II for Illumina (E7370S, NEB). DNA libraries were validated again on the Agilent TapeStation 4150, and were quantified by qPCR (E7630, NEB). The 2 × 150 bp paired-end sequencing with average insert size of 700 bp was performed by Welgene Biotech on an Illumina NovaSeq 6000 platform.

WGS data analysis

Since there was no assembled *S. peruvianum* genome, high quality Illumina reads were mapped to the *S. lycopersicum* Heinz 1706 reference genome (SL4.0) (Hosmani et al., 2019) by the GPU-based NVIDIA Clara Parabricks package (NVIDIA). To determine the variant frequency, we used the deep learning-based Google DeepVariant (Yun et al., 2021) with "WGS model" to identify variants. All samples were then combined by GLnexus (Yun et al., 2021) to perform "joint genotype calling" using "DeepVariant" model to combine samples. We then calculated the heterozygous allele frequency by dividing the read depth of the heterozygous allele (labeled as 0/1 by GLnexus) over the total read depth of the variant. A large chromosomal region with heterozygous allele frequency lower than 0.5 indicated either a chromosome region with low recombination rate or deletion of the chromosome fragments. To determine CNVs between samples, we used the cn.mops pipeline (Klambauer et al., 2012) to analyze mapped Illumina reads. To minimize the effects of repetitive sequence regions, we set the segment size to 3,000 bp and minimum number of segments as 10 to identify high confidence CNVs.

Reverse transcription quantitative PCR

Expression of four genes was analyzed using reverse transcription quantitative PCR (RT-qPCR). These genes were: *SpSGS3*, *SpARF3*, *SpARF4*, and *SpRDR6*. RT-qPCR analysis was performed on three biological replicates and three replicates in a CFX Connect instrument (Bio-Red, Hercules, CA, USA). RT-qPCR was carried out in 96-well optical reaction plates using the iQ SYBR Green Supermix (Bio-Rad). The reference gene Actin and gene-specific primers for the RT-qPCR are listed in Supplemental Table S8.

Data availability statement

The Illumina sequencing reads generated for this study have been deposited at NCBI under BioProject PRJNA768623.

Accession numbers

Sequence data from this article can be found in Supplemental Table S9.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of cytokinins on callus induction (first subculture) and callus proliferation (second subculture).

Supplemental Figure S2. Effects of cytokinins on callus in solid medium (third 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.

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