Generation and Molecular Characterization of CRISPR/Cas9-Induced Mutations in 63 Immunity-Associated Genes in Tomato Reveals Specificity and a Range of Gene Modifications

Ning Zhang[1†], Holly M. Roberts[1†], Joyce Van Eck[1,2†] and Gregory B. Martin[1,3*†]

[1] *Boyce Thompson Institute for Plant Research, Ithaca, NY, United States,* [2] *Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, United States,* [3] *Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, United States*

The CRISPR/Cas9 system is a powerful tool for targeted gene editing in many organisms including plants. However, most of the reported uses of CRISPR/Cas9 in plants have focused on modifying one or a few genes, and thus the overall specificity, types of mutations, and heritability of gene alterations remain unclear. Here, we describe the molecular characterization of 361 T0 transgenic tomato plants that were generated using CRISPR/Cas9 to induce mutations in 63 immunity-associated genes. Among the T0 transformed plants, 245 carried mutations (68%), with 20% of those plants being homozygous for the mutation, 30% being heterozygous, 32% having two different mutations (biallelic), and 18% having multiple mutations (chimeric). The mutations were predominantly short insertions or deletions, with 87% of the affected sequences being smaller than 10 bp. The majority of 1 bp insertions were A (50%) or T (29%). The mutations from the T0 generation were stably transmitted to later generations, although new mutations were detected in some T1 plants. No mutations were detected in 18 potential off-target sites among 144 plants. Our study provides a broad and detailed view into the effectiveness of CRISPR/Cas9 for genome editing in an economically important plant species.

Keywords: CRISPR/Cas9, genome editing, immunity-associated genes, tomato, Off-target mutation

INTRODUCTION

Derived from a native adaptive immune system in eubacteria and archaea, the CRISPR/Cas system enables the alteration of DNA sequences in many organisms to achieve precise gene modifications (Jaganathan et al., 2018). The most widely used *Streptococcus pyogenes* Cas9 (SpCas9) requires the 20-bp spacer sequence of a guide RNA (gRNA) to recognize a complementary target DNA site upstream of a protospacer adjacent motif (PAM) and generates a double-stranded break (DSB) near the target

region (Xie and Yang, 2013). DSBs are repaired through either non-homologous end joining (NHEJ) or homology-directed recombination (HDR) resulting in small insertions/deletions (indels) or substitutions at the target region, respectively (Jinek et al., 2012). Compared to other genome editing tools such as zinc finger nucleases (ZFNs; Kim et al., 1996) and transcription activator-like effector nucleases (TALENs; Bogdanove and Voytas, 2011), CRISPR/Cas is more robust in that the Cas protein can theoretically bind to any genomic region preceding a PAM site and, importantly, target multiple sites simultaneously. However, the possibility of off-target mutations caused by CRISPR/Cas is a potential concern in both basic and applied research in plants, although it has been reported that off-target effects of CRISPR/Cas occur at a much lower frequency in plants than in mammals (Fu et al., 2013; Kuscu et al., 2014). The most effective way to minimize off-target mutations is to select a gRNA target with little or no homology to other genomic regions (Baltes and Voytas, 2015). Other methods to reduce off-target mutations include using paired Cas9 nickases (Ran et al., 2013) or paired fusions of a catalytically dead Cas9 nuclease to the *Fok*I cleavage domain (Guilinger et al., 2014; Tsai et al., 2014).

As one of the most important vegetable crops in the world (Kimura and Sinha, 2008), tomato (*Solanum lycopersicum*) is an important source of health-promoting nutrients including vitamin C and E, minerals, and carotenes such as ß-carotene and lycopene (Canene-Adams et al., 2005). However, tomato production is threatened by many infectious diseases, including bacterial speck disease caused by *Pseudomonas syringae* pv. tomato (*Pst*), which can result in severe economic losses due to reduced yield and quality (Xin and He, 2013). A large number of candidate immunity-associated genes have been identified in tomato, but validation of the functional importance of these genes had been technically challenging before the emergence of CRISPR/Cas technology (Pedley and Martin, 2003; Oh and Martin, 2011; Rosli et al., 2013; Pombo et al., 2014). Although CRISPR/Cas has been used to modify genes with key roles in growth, development, and biotic and abiotic stresses in plants (Ito et al., 2015; Jacobs et al., 2017; Nekrasov et al., 2017; Rodriguez-Leal et al., 2017; Shimatani et al., 2017; Yu et al., 2017; D'Ambrosio et al., 2018; Hashimoto et al., 2018; Li et al., 2018a; Li et al., 2018b; Tashkandi et al., 2018; Zsogon et al., 2018; Ortigosa et al., 2019; Wang et al., 2019; Xu et al., 2019), all of the reported studies have focused on one or a few genes and could not provide broad insights into the specificity, types of mutations and heritability of genome editing by CRISPR/Cas9 in tomato.

Recently, we developed the Plant Genome Editing Database (PGED; http://plantcrispr.org/cgi-bin/crispr/index.cgi) which provides information about a collection of tomato lines with CRISPR/Cas9-induced mutations in immunity-associated genes (Zheng et al., 2019). In the present study, we molecularly characterized 361 T0 transgenic tomato plants that were generated using CRISPR/Cas9 to induce mutations in 63 candidate immunity-associated genes. To enhance the mutation efficiency and reduce the number of transformations needed, we evaluated gRNA efficiency by transient expression in tomato leaves and conducted tomato transformation with *Agrobacterium* pools containing 2–4 Cas9/gRNA constructs. This initial evaluation of gRNAs allowed us to select the most efficient ones for tomato stable transformation while using "agrobacterium pools" with various Cas9/gRNAs constructs shortened the time for generating multiple tomato mutant lines. We established an efficient CRISPR/Cas9 system to generate a large number of primary transgenic lines and we report for the first time a systematic investigation of the specificity of targeting, the types of mutations generated and the heritability of the mutations through multiple generations of tomato. Our CRISPR/Cas9-induced tomato mutant plants provide a powerful resource for better understanding the molecular mechanisms of plant-microbe interactions in the future.

MATERIALS AND METHODS

Guide RNA (gRNA) Design and Construct Development

All 20-nt gRNAs specific for the target genes were designed using the software Geneious R11 as described previously (Jacobs et al., 2017). The tomato (*Solanum lycopersicum*) reference genome sequence (SL2.5 or SL3.0) was used as an off-target database to score each gRNA (GN_{19} or gN_{19}; "g" represents a manually added "G" to accommodate the transcription initiation requirement of the U6 promoter if the first nucleotide is not a G at target sites) preceding a PAM (NGG) sequence. For each gene, 2–3 gRNA targets with minimum off-target scores were designed. Single or multiple gRNA cassettes were cloned into a binary vector p201N: Cas9 by Gibson assembly as described previously (Jacobs and Martin, 2016). Colonies containing correct gRNA sequences were confirmed by PCR and Sanger sequencing.

Evaluation of gRNA Efficiency by Agroinfiltration in Tomato and *Nicotiana benthamiana* Leaves

Each Cas9/gRNA vector was transformed into the *Agrobacterium tumefaciens* strain 1D1249 (Wroblewski et al., 2005) by electroporation. For agroinfiltration into tomato leaves, the bacterial cells containing different gRNA plasmids were grown in liquid YEP medium with 150 mg/L kanamycin overnight at 30°C. The bacterial pellet was collected and resuspended in an induction buffer containing 10 mM $MgCl_2$, 10 mM MES (pH 5.7), and 200 µM acetosyringone (Sigma-Aldrich). Bacterial suspensions were adjusted to $OD_{600} = 0.3$ and incubated at room temperature for 2–5 h. The third and fourth leaves of 4-week-old tomato plants were infiltrated with needle-less syringes and the whole plant was then placed in a growth chamber with a temperature of 22°C–24°C, 16 h light/8 h dark photoperiod and 65% relative humidity. Three days later, a pool of six leaf discs were collected from three individual plants (two leaf discs from each of three plants) that had been infiltrated with the tested Cas9/gRNA vector, and used for genomic DNA extraction, PCR and sequencing. The web-based tool TIDE (https://tide.deskgen.com) was used to determine the mutation frequency induced by corresponding Cas9/gRNA vectors.

Tomato Transformation

Tomato transformation was performed either at the plant transformation facility at the Boyce Thompson Institute (BTI) or North Carolina State University (NCSU) (Gupta and Van Eck, 2016; Van Eck et al., 2019). Modifications of the transformation methods were made for Rio Grande (RG), including using 100 mg/L kanamycin for selection, and adding 0.1 mg/L indole-3-acetic acid (IAA) to the plant regeneration media (2Z, 1Z) and 1 mg/L IAA to the rooting medium. Each Cas9/gRNA vector was first electrotransformed into *Agrobacterium tumefaciens* LBA4404 (BTI), AGL1 (BTI), or GV3101(pMP90) (NCSU). In most cases, 2–4 *Agrobacterium* culture preparations (of the same *Agrobacterium* strain), each carrying a different Cas9/gRNA construct, were pooled together and used for transformation to minimize the number of experiments. Tomato genotypes RG-PtoR or RG-prf3 were used for transformation if not specifically labeled (**Table 1**).

Genotyping and Mutation Analysis

Genomic DNA was extracted from tomato cotyledons or young leaves using a modified CTAB method (Murray and Thompson, 1980). The existence of T-DNA was confirmed by PCR using primers listed in **Table S6**. To determine the mutation specificity, genomic regions flanking the target site of each gene were amplified with specific primers (see PGED; http://plantcrispr.org/) and sequenced by Sanger sequencing. TIDE was used to rapidly evaluate the mutated allelic sequences using the sequencing files (.ab1 format), especially for PCR amplicons of biallelic, heterozygous, or chimeric mutants whose mutation length and frequency cannot be determined due to superimposed sequencing chromatograms.

Off-Target Evaluation

To evaluate potential off-target mutations caused by gRNAs in CRISPR-induced mutant plants, twelve gRNAs were selected and used as queries to search for potential off-target sites across the tomato genome with up to 4-nt mismatches and 1-nt indel by the software Geneious R11 or with up to 3-nt mismatches by a web tool Cas-OFFinder. Each off-target site was given a score based on how similar it was to the spacer sequence of gRNAs. A higher score for an off-target site indicated a higher similarity to the original target site and a higher likelihood to cause off-target mutations. A shortlist of potential off-target sites of each gRNA queried was generated by selecting their relatively high-scoring off-target sites predicted by either Geneious R11 or Cas-OFFinder (**Table 3**). Similar to mutation genotyping described above, genomic regions flanking the putative off-target sites were amplified with specific primers (**Table S7**) and PCR amplicons were sequenced to detect if off-target mutations were induced in those regions.

RESULTS

CRISPR/Cas9 Targeting of Immunity-Associated Genes in Tomato

To study the efficiency and specificity of genome editing in tomato by CRISPR/Cas9 and to better understand plant-pathogen interactions, we generated a collection of tomato lines with targeted CRISPR/Cas9-induced mutations in genes that have been implicated in the immune response. Candidate genes were selected based on previous studies involving RNA-Seq, biochemical approaches, virus-induced gene silencing (VIGS), or yeast two-hybrid (Y2H) screens (Zeng et al., 2012; Rosli et al., 2013; Pombo et al., 2014; Giska and Martin, 2019); orthologs of immunity-associated genes reported in other plant species such as Arabidopsis and rice were also included (Shimizu et al., 2010; Hutin et al., 2015; Xin et al., 2016; Yamada et al., 2016; Stegmann et al., 2017).

For each candidate gene, at least two gRNAs targeting different DNA sites were designed and separately cloned into a Cas9-expressing binary vector p201N:Cas9 (Jacobs and Martin, 2016). The gRNAs were designed to be highly specific at target sites and their predicted off-target sites contained at least one nucleotide mismatch in the seed sequence (the last 12 nucleotides preceding the PAM) or two nucleotide mismatches against the full 20-bp protospacer (although some gRNAs were designed to intentionally modify multiple homologs simultaneously). Most Cas9/gRNA constructs in this study had only one gRNA expression cassette per plasmid, except one construct that contained three gRNA cassettes targeting three *Aquaporin transporter* genes (**Table 1**).

Evaluation of gRNA Effectiveness by Agroinfiltration in Tomato and *Nicotiana benthamiana* Leaves

To initially evaluate the effectiveness of gRNAs and subsequently enhance the mutation rate in stably transformed tomato plants, 195 gRNAs were tested for their ability to cause mutations using *Agrobacterium* infiltration (agroinfiltration) in tomato leaves (**Figure 1A**; **Table S1**). After agroinfiltration, DNA was isolated from the leaf tissue and the genomic region spanning each target site was PCR amplified, sequenced, and analyzed with a web-based tool called Tracking of Indels by Decomposition (TIDE; https://tide.deskgen.com; Brinkman et al., 2014) to calculate the mutation frequency. gRNAs with mutation frequency >0 (p < 0.0001) were considered to be effective in inducing mutations in this assay while those with mutation rate = 0 were considered ineffective. A total of 61.5% of the tested gRNAs were effective in inducing transient mutations in tomato leaves (**Figure 1B**). Among these, 96% had a mutation rate greater than 0% but less than 10% in this assay, while only five gRNAs (4%) had a mutation frequency over 10% (**Figure 1C**).

Agroinfiltration of tomato leaves is not very efficient and to test whether this affected our estimate of gRNA mutation efficacy, we evaluated the mutation efficiency of two of the gRNAs (Bti9ab-1 and Drm3-1) which each have identical target sites in both tomato and *Nicotiana benthamiana* (**Figure S1**). The mutation frequency induced by these two gRNAs in tomato was much lower than in *N. benthamiana* (**Figure S1A**). In addition to these two gRNAs, we also tested another four gRNAs that each target two of the four homologs of the *Mai1* gene in *N. benthamiana* (Roberts et al., 2019; **Figure S1B**). In *N. benthamiana*, the majority of the gRNA targets showed a

TABLE 1 | Mutation rates and mutation types in T0 transgenic plants. See also **Table S3**.

Target genes	Solyc #	# of transgenic plants	# of edited plants	Mutation rate (%)[a]	Mutation types[b]
ADE	Solyc05g005700	10	5	50	3 homo; 1 hetero; 1 chimeric
AOX	Solyc08g075550	9	8	89	2 homo; 2 biallelic; 3 hetero; 2 chimeric
APE	Solyc11g018800	1	1	100	1 biallelic
Aquaporin 1/2/3[c]	Solyc11g069430 (Aquaporin 1) Solyc06g074820 (Aquaporin 2) Solyc08g066840 (Aquaporin 3)	12	10	83	5 homo; 2 biallelic; 10 hetero; 2 chimeric
BHLH	Solyc03g114230	3	3	100	3 biallelic
BSK830	Solyc12g099830	8	6	75	2 biallelic; 3 hetero; 1 chimeric
BSK830 (Hawaii 7981)[d]	Solyc12g099830	6	5	83	3 biallelic; 1 hetero; 1 chimeric
Bti9-interactor	Solyc09g008010	1	1	100	1 homo
Bti9ab	Solyc07g049180	4	3	75	2 homo; 1 biallelic
CathepsinB1	Solyc02g076980	29	20	69	8 homo; 8 biallelic; 2 hetero; 2 chimeric
CathepsinB2	Solyc02g077040	11	8	73	2 homo; 3 biallelic; 3 hetero
CORE	Solyc03g096190	7	6	86	1 homo; 4 biallelic; 1 hetero
Drm-3	Solyc01g099840	3	3	100	2 biallelic; 1 hetero
EDS1	Solyc06g071280	2	2	100	1 homo; 1 chimeric
ERF5	Solyc03g093560	7	5	71	2 biallelic; 1 hetero; 2 chimeric
Fen	Solyc05g013290	18	13	72	5 biallelic; 7 hetero; 1 chimeric
Fen (RG-pto11)[e]	Solyc05g013290	15	9	60	2 homo; 4 biallelic; 5 chimeric
Fls2.1	Solyc02g070890	4	4	100	3 homo; 1 hetero
Fls3	Solyc04g009640	11	7	64	5 homo; 2 hetero
LRRXII-1	Solyc06g076910	5	1	20	1 biallelic
LRRXII-2	Solyc04g012100	4	3	75	1 biallelic; 2 hetero
Mai1	Solyc04g082260	4	1	25	1 hetero
Mai5	Solyc10g085990	11	8	73	2 homo; 5 hetero; 1 chimeric
MAP3Ka	Solyc11g006000	6	6	100	4 biallelic; 2 hetero
Min7	Solyc12g017830	5	4	80	1 biallelic; 1 hetero; 2 chimeric
MKK1	Solyc12g009020	2	2	100	1 biallelic; 1 chimeric
MKK2	Solyc03g123800	6	5	83	1 homo; 4 biallelic
MKK4	Solyc03g097920	2	1	50	1 hetero
MKKK15	Solyc02g065110	8	6	75	4 hetero; 2 chimeric
MKKK66	Solyc08g081210	3	1	33	1 hetero
MLO16	Solyc11g069220	4	4	100	1 biallelic; 2 hetero; 1 chimeric
NOD	Solyc11g008200	8	7	88	1 homo; 4 hetero; 2 chimeric
NPR1	Solyc07g040690	5	1	20	1 hetero
NRC1/2/3[f]	Solyc01g090430 (NRC1) Solyc10g047320 (NRC2) Solyc05g009630 (NRC3)	8	5	63	1 homo; 3 biallelic; 1 hetero
PAD4	Solyc02g032850	7	1	14	1 biallelic
PBCP	Solyc03g116690	3	2	67	1 homo; 1 biallelic
PBL-T1	Solyc09g007170	5	3	60	1 biallelic; 2 chimeric
PBL-T2	Solyc01g067400	2	2	100	2 hetero
Peptide Transporter 3	Solyc05g009500	4	3	75	1 biallelic; 1 hetero; 1 chimeric
Permease Transporter	Solyc03g005820	4	2	50	1 homo; 1 biallelic
PGA1[f]	Solyc05g005560 Solyc05g005570	5	2	40	2 hetero
Pic1	Solyc07g066260	5	4	80	1 biallelic; 2 hetero; 1 chimeric
PR1b	Solyc00g174340	6	4	67	3 biallelic; 1 chimeric
Propep1	Solyc04g072310	2	2	100	2 chimeric
RALF1	Solyc01g067900	3	3	100	1 homo; 2 biallelic
RALF2	Solyc01g099520	10	7	70	1 homo; 3 hetero; 3 chimeric
RbohB	Solyc03g117980	8	3	38	1 homo; 2 biallelic
SAG101-1/2[f]	Solyc02g069400 (SAG101-1) Solyc02g067660 (SAG101-2)	3	2	67	1 homo; 1 biallelic
SlMlo1/9[f]	Solyc04g049090 (SlMlo1) Solyc06g010030 (SlMlo9)	10	9	90	1 homo; 2 biallelic; 3 hetero; 6 chimeric
SOBIR/SOBIR-like[f]	Solyc06g071810 (SOBIR) Solyc03g111800 (SOBIR-like)	16	9	56	2 homo; 1 biallelic; 4 hetero; 3 chimeric
Solute Transporter 2	Solyc05g005950	4	2	50	1 biallelic; 1 hetero

(Continued)

TABLE 1 | Continued

Target genes	Solyc #	# of transgenic plants	# of edited plants	Mutation rate (%)[a]	Mutation types[b]
STP13	Solyc09g075820	2	1	50	1 homo
TFT1	Solyc11g010470	3	3	100	1 biallelic; 2 hetero
TFT10	Solyc04g076060	4	2	50	2 chimeric
TFT7	Solyc04g074230	2	2	100	2 hetero
Wak1	Solyc09g014720	5	5	100	2 homo; 2 biallelic; 1 chimeric
WRKY11	Solyc08g006320	2	2	100	2 chimeric
WRKY9b	Solyc08g067360	4	1	25	1 biallelic

[a]*Mutation rate (%) = number of plants with mutations/number of total transgenic plants. Mutations were analyzed by Geneious R11 and TIDE.* [b]*Homo, homozygous mutation; hetero, heterozygous mutation.* [c]*Three gRNA cassettes were cloned into one p201N:Cas9 plasmid.* [d]*Tomato genotype Hawaii 7981 was used for transformation.* [e]*Tomato genotype RG-pto11 was used for transformation.* [f]*Specific gRNA for each gene was independently cloned into the p201:Cas9 vector and the two or three gRNA/Cas9 constructs were pooled together for tomato transformation.*

mutation frequency of 10%–40%, while a small number had a mutation frequency less than 5% (**Figure S1B, C**). These observations suggest that inefficiency of agroinfiltration in tomato leaves probably leads to an underestimate of the true efficacy of gRNAs for generating mutations. This is supported by later observations in which some low-efficient gRNAs were very effective in inducing mutations in stably-transformed tomato plants (**Table S2**). Factors that contribute to this lower activity of gRNAs by agroinfiltration of tomato leaves are unknown but might include possible specific immune responses of tomato against *Agrobacterium* or less efficient infiltration due to leaf morphology differences with *N. benthamiana*.

In most cases, we selected the most efficient gRNAs for subsequent stable transformation in tomato, however, some low-efficiency gRNAs were also used if limited gRNAs could be designed for a particular target gene. Most gRNAs that were effective in inducing mutations in the agroinfiltration transient assay also induced mutations in stable transgenic seedlings, with one exception where a gRNA that had a 4.4% mutation frequency in the transient assay did not edit target genes in two stably-transformed plants (**Table S2**). It was not possible, however, to directly compare gRNA efficiency in the transient assay with that in stable transformation, as other factors such as the bias of gRNA transformation into plants using "*Agrobacterium* pools" and the total number of regenerated transgenic seedlings varied from gene to gene in stable transformation.

CRISPR/Cas9-Induced Mutations in T0 Transgenic Plants

A total of 361 putative primary (T0) transgenic tomato plants were regenerated by *Agrobacterium tumefaciens*-mediated stable transformation. To confirm the mutated sequence(s) in each plant, genomic regions spanning the target sites were PCR amplified and sequenced. All five possible genotypes, that is, wild-type, homozygous for the mutation, biallelic (a different mutation in each allele), heterozygous for the mutation, or multiple mutations (chimeric), were detected in our stably transformed tomato plants (**Table 1**). Direct sequencing of PCR amplicons containing biallelic, heterozygous, or chimeric mutations resulted in superimposed sequencing chromatograms, which made it difficult to determine specific mutation types and mutation frequency in those plants. To resolve this problem, TIDE was used to rapidly determine the mutated allelic

sequences using the sequencing file (.ab1 format) with superimposed chromatograms (Brinkman et al., 2014), thus avoiding tedious and expensive cloning and multi-clone sequencing for mutation analysis.

Of the 361 T0 plants, 245 were found to have modifications at the target site(s) within 63 genes (**Table 1**; **Table S3**). Most of the lines had only one CRISPR-induced mutation in one gene per plant, while a few had mutations in two or even three genes (the latter cases occurred when using *Agrobacterium* pooling – see below). We identified only one mutant event for some of the targeted genes while for others up to 20 independent mutant events were generated (**Table 1**). Overall, the average editing efficiency (the number of edited plants/the number of transgenic plants) by CRISPR/Cas9 in tomato in our experiments was 68% (**Figure 2A**), although the mutation rate varied over a wide range from 14% to 100% from target gene to target gene in different mutant lines (**Table 1**). All four mutation types (homozygous, biallelic, heterozygous, or chimeric) were observed in several mutant lines that had sufficient independent mutant events, while there was a bias of mutation types in some mutant lines, probably due to the limited number of transgenic events generated (**Table 1**).

We analyzed the distribution of the four mutation types in all the 245 T0 plants at the 267 mutated target sites (some plants had more than one target site) and found that the percentage of homozygous, biallelic, heterozygous, or chimeric mutation was 20%, 32%, 30%, and 18%, respectively (**Figure 2B**). In particular, plants having homozygous or biallelic mutations accounted for 52% of the total. These mutants and their progenies can be used directly for phenotype screening because no wild-type alleles are present, thus speeding the research process by saving time for further genotyping in the next generation. The most common mutation alterations induced by CRISPR/Cas9 were deletions or insertions, with 87% of these modifications at the target sites being less than 10 bp (**Figure 2C**). The proportion of deletion mutations was 77%, and the deletion length spanned a wide range from 1 bp to over 400 bp. Of all the mutations, the most abundant modification was 1-bp deletion or insertion (**Figure 2C**). For these, A- and T-insertions accounted for 79.5%, while G-insertions accounted for only 4.5% (**Figure 2D**). Base substitutions in combination with indels were also detected, but at a much lower frequency. Only three independent mutant events (two were the same mutation type) harbored a nucleotide
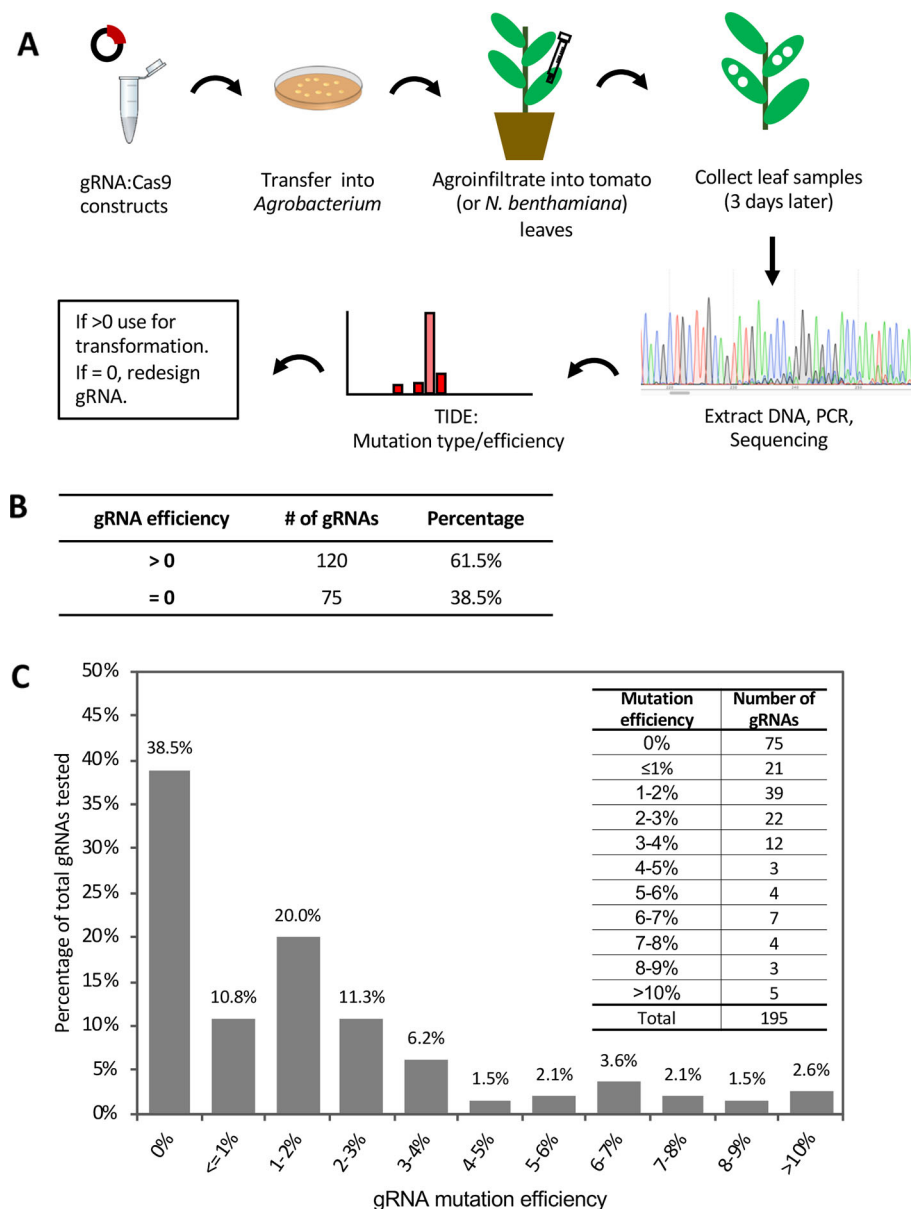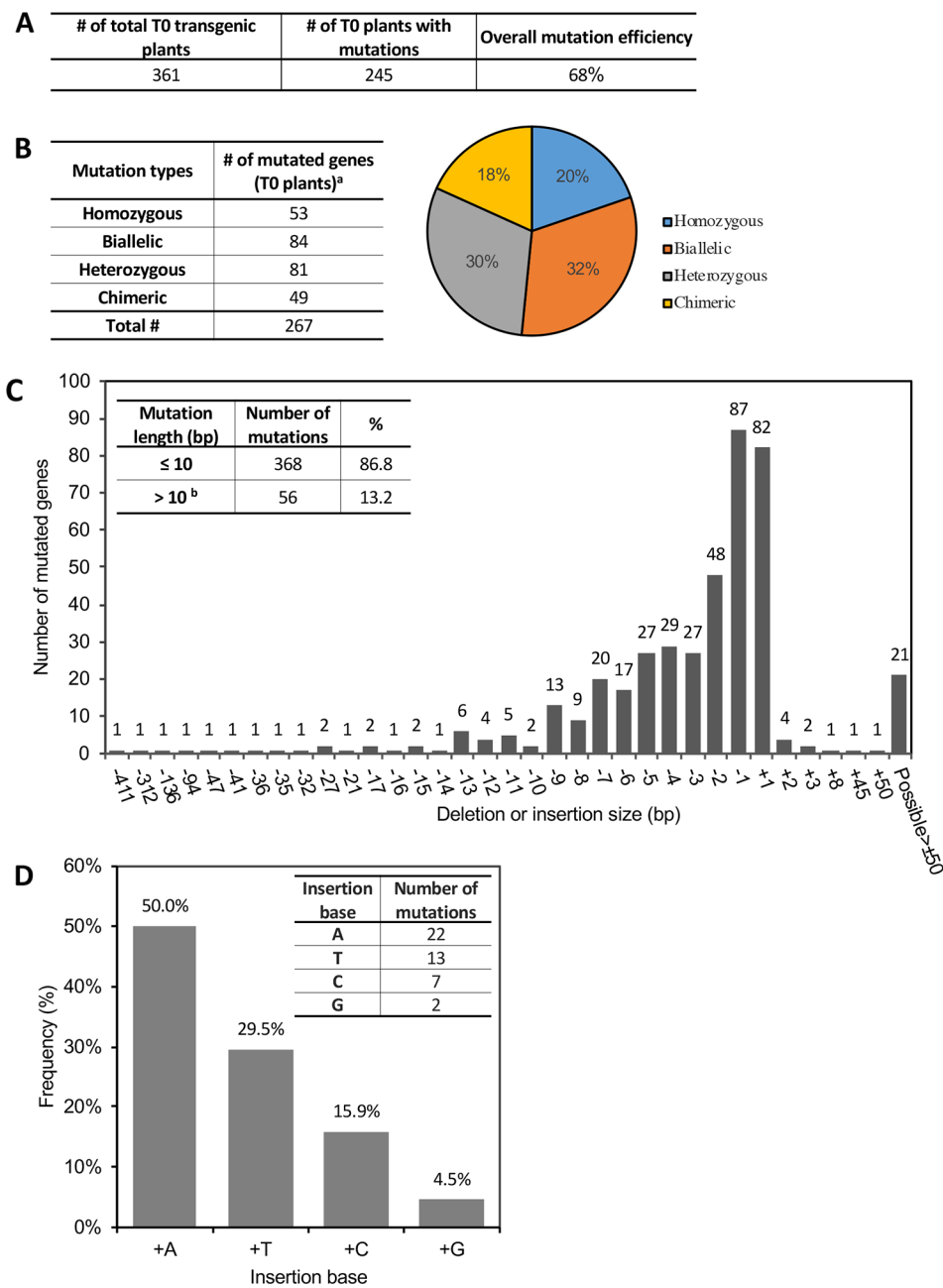
FIGURE 1 | Evaluation of gRNA-mediated mutation efficiency by agroinfiltration in tomato leaves. See also **Figure S1** and **Table S1**. **(A)** Schematic showing the workflow of guide RNA (gRNA) evaluation by agroinfiltration. **(B)** Summary of gRNA efficiency tested by agroinfiltration. **(C)** The distribution of mutation efficiencies of the 195 gRNAs. Inset on the top right shows the number of gRNAs in each mutation efficiency range. TIDE (https://tide.deskgen.com) was used to calculate mutation efficiency by identifying the predominant types of insertions and deletions (indels) in the DNA of a targeted cell pool.

substitution in one copy of the target genes at the positions 5-bp preceding the PAM, along with a short insertion or deletion at the target site (**Figure S2**).

Multiplex editing of three *Aquaporin transporter* (*AquaT*) genes by using one Cas9/gRNA construct was also tested. Three individual promoter-gRNA expression cassettes (in the order *Aqua1-Aqua2-Aqua3*) were assembled into the p201N:Cas9 vector (**Figure S3A**) as previously reported (Jacobs et al., 2017). Ten of the 12 regenerated transgenic plants were edited, including

three single mutants, four double mutants, and three triple mutants (having mutations in all three genes). Interestingly, all the three single mutants knocked out *AquaT1*, while three double mutants modified *AquaT1* and *AquaT2* and one edited *AquaT1* and *AquaT3* simultaneously. Three plants had mutations in all the three *AquaT* genes together (**Figure S3B**). These data suggested the position of the gRNA cassette in the vector may affect its mutation rate, considering that all gRNAs were efficient enough to induce mutations once transformed into plants.

A

# of total T0 transgenic plants	# of T0 plants with mutations	Overall mutation efficiency
361	245	68%

B

Mutation types	# of mutated genes (T0 plants)[a]
Homozygous	53
Biallelic	84
Heterozygous	81
Chimeric	49
Total #	267

Pie chart: Homozygous 20%, Biallelic 32%, Heterozygous 30%, Chimeric 18%

C

Mutation length (bp)	Number of mutations	%
≤ 10	368	86.8
> 10 [b]	56	13.2

Bar chart — Number of mutated genes vs Deletion or insertion size (bp):
-411: 1, -312: 1, -136: 1, -94: 1, -47: 1, -41: 1, -36: 1, -35: 1, -32: 1, -27: 2, -21: 1, -17: 2, -16: 1, -15: 2, -14: 1, -13: 6, -12: 4, -11: 5, -10: 2, -9: 13, -8: 9, -7: 20, -6: 17, -5: 27, -4: 29, -3: 27, -2: 48, -1: 87, +1: 82, +2: 4, +3: 2, +8: 1, +45: 1, +50: 1, Possible>±50: 21

D

Insertion base	Number of mutations
A	22
T	13
C	7
G	2

Bar chart — Frequency (%) vs Insertion base:
+A: 50.0%, +T: 29.5%, +C: 15.9%, +G: 4.5%

FIGURE 2 | CRISPR/Cas9-induced gene mutations in T0 transgenic plants. See also **Table S3**. **(A)** The average mutation rate induced by CRISPR/Cas9 in T0 plants. **(B)** Summary of CRISPR-induced mutation types and their frequency in T0 plants. Left: Number of genes modified with the corresponding mutation type; Right: Percentage of genes harboring the corresponding mutation type. [a] Some plants have multiple target genes in one plant. **(C)** Frequency of each insertion or deletion mutation. x-axis: number of base pairs (bp) deleted (–) or inserted (+) into target sites. Inset on the left top shows the percentage of mutations ≤10 bp or >10 bp. [b]All "possible > ± 50 bp" in the figure are included in >10 bp. TIDE only calculates mutation length ≤50 bp. **(D)** Percentage of different bases in the 1-bp insertion mutations. Inset at the right top shows the number of mutations with each type of inserted base.

Heritability of the Mutations

To evaluate the heritability of mutations in T0 plants, a large number of T1 and some T2 plants were generated and examined for their genotypes at the target sites. Most of the same genotypes from T0 plants were transmitted to plants in later generations, although we did not record segregation ratios in the progenies.

Of note, no new mutations or reversions to wild-type were found in the progeny of any homozygous T0 plants, indicating all the homozygous mutations occurred in the transformed embryogenic cells before the first division. However, we did observe novel genotypes in a small percentage of T1 or T2 plants whose progenitor (T0 plants) harbored biallelic, heterozygous or

chimeric mutations (**Table 2**). In particular, a homozygous mutation (−265 bp) was detected in the progeny of a NRC2 primary transgenic plant with a "+1 bp/+2 bp" biallelic mutation. It is possible that the new −265 bp modification at the target site was created by further modification of the existing +1 bp or +2 bp mutations in the progenitor as has been reported in rice and carrot (Klimek-Chodacka et al., 2018; Tang et al., 2018). It is also possible that the biallelic T0 plant (+1 bp/+2 bp) was a chimera and the new mutation derives from chimeric tissue of the T0 plant (Zhang et al., 2014).

We also observed new genotypes in the progeny of some T0 plants harboring heterozygous or chimeric mutations (**Table 2**) even though most of the progeny still possessed the same genotypes as the progenitor line. In some of these cases, CRISPR/Cas9 continued to modify the wild-type allele of the target gene in the progeny if the parent plants still contained a wild-type allele and the Cas9/gRNA expression cassette (**Table 2**). In other cases, unexpected genotypes were detected in some mutant lines including Mai1-E10, CathepsinB2-E8, Min7-E6, and ADE-E2 (**Table 2**). For instance, although the ADE-E2 T0 plant was chimeric (−4 bp/−5 bp/−13 bp) without a wild-type allele, we identified one T1 plant that was azygous (two copies of wild-type allele) and another one with a novel biallelic mutation (−4bp/−9 bp). Another example is the CathepsinB2-E8

T0 plant which had a heterozygous (−4 bp/WT) mutation. However, −2 bp homozygous and −1 bp/WT heterozygous mutations were detected in later generations. The unexpected new genotypes discussed above revealed that the one leaf/ cotyledon sample may not reveal all the genotypes in the whole plant if the mutant is chimeric. Therefore, for T0 edited plants without any wild-type allele, it will still be useful to perform genotyping in subsequent generations to obtain homozygous mutants without the presence of Cas9/gRNA.

Specificity of CRISPR-Induced Gene Modifications in Tomato

Mutations in unintended sequences (off-target mutations) is a possible concern in both functional genomics studies and plant breeding. To evaluate potential off-target effects by CRISPR/Cas9 in our tomato lines, we first evaluated the specificity of 12 gRNAs of Cas9 by Geneious R11 (https://www.geneious.com; Kearse et al., 2012) and Cas-OFFinder (Bae et al., 2014). The putative off-target sites predicted by Cas-OFFinder were then manually checked using JBrowse (https://solgenomics.net/jbrowse_ solgenomics/) to confirm their locations in the tomato genome. The presence of a PAM was required for the site to be considered a candidate site. These gRNAs were selected for off-target

TABLE 2 | New genotypes detected in T1 or T2 plants.

T0 mutation type[a]	Generation	Plants	Gene Solyc#	Mutation[b]	Is T-DNA present?
Bialleleic	**T0**	**NRC2-E4**	**Solyc10g047320**	**+1 bp/+2 bp**	**Yes**
	T1	NRC2-E4-P2	Solyc10g047320	+1 bp/large deletion[c]	No
	T2	NRC2-E4-P2-2	Solyc10g047320	-265 bp/-265 bp	No
	T2	NRC2-E4-P2-4	Solyc10g047320	+1 bp/+1bp	No
Heterozygous	**T0**	**Mai1-E10**	**Solyc04g082260**	**-4 bp/WT**	**Yes**
	T1	Mai1-E10-P20	Solyc04g082260	+1 bp/WT	Yes
Heterozygous	**T0**	**CathepsinB2-E8**	**Solyc02g077040**	**-4 bp/WT**	**Yes**
	T1	CathepsinB2-E8-P2	Solyc02g077040	-2 bp/-2 bp	No
	T1	CathepsinB2-E8-P10	Solyc02g077040	-1 bp/WT	No
Heterozygous	**T0**	**Min7-E6**	**Solyc12g017830**	**+1 bp/WT**	**Yes**
	T1	Min7-E6-P4	Solyc12g017830	+1 bp/-3 bp	Yes
	T1	Min7-E6-P8	Solyc12g017830	-1 bp/WT	No
Heterozygous	**T0**	**NRC2-E1**	**Solyc10g047320**	**-3 bp/WT**	**Yes**
	T1	NRC2-E1-P6	Solyc10g047320	-3 bp/-5 bp	Yes
	T1	NRC2-E1-P15	Solyc10g047320	-3 bp (58%); -6 bp (32%) +1 bp (3.6%)	No
Chimeric	**T0**	**MKKK15-E2**	**Solyc02g065110**	**-1 bp (10.4%); +1 bp (2.1%); WT (85%)**	**Yes**
	T1	MKKK15-E2-P2	Solyc02g065110	-5 bp(60%); -1 bp (31%); +1 bp (4.7%)	Yes
	T1	MKKK15-E2-P3	Solyc02g065110	-5 bp (20%); WT (74%)	No
	T1	MKKK15-E2-P7	Solyc02g065110	+1 bp (77.6%); -5 bp (4.7%); WT (9.5%)	Yes
Chimeric	**T0**	**MKK1-E1**	**Solyc12g009020**	**-7 bp (8.1%); -2 bp (33.3%); WT (48.5%)**	**Yes**
	T1	MKK1-E1-P3	Solyc12g009020	-2 bp/-4 bp	No
Chimeric	**T0**	**Min7-E5**	**Solyc12g017830**	**-1 bp (35.9%); +1 bp (9%); WT (49.4%)**	**Yes**
	T1	Min7-E5-P1	Solyc12g017830	-1 bp/-3 bp	Yes
	T1	Min7-E5-P2	Solyc12g017830	-6 bp (64.5%); +1 bp (19%); -1 bp (8.8%); -7 bp (3.4%)	Yes
	T1	Min7-E5-P3	Solyc12g017830	-5 bp/-6 bp	Yes
	T1	Min7-E5-P7	Solyc12g017830	-9 bp (71.3%); +1 bp (18.2%); -1 bp (3.6%)	Yes
Chimeric	**T0**	**ADE-E2**	**Solyc05g005700**	**-4 bp (20%); -5 bp (40.4%); -13 bp (13.9%)**	**Yes**
	T1	ADE-E2-P1	Solyc05g005700	WT/WT	No
	T1	ADE-E2-P20	Solyc05g005700	-4 bp/-9 bp	No

[a]For each mutant event, only the plants harboring different genotypes from their T0 progenitors are listed. Unlisted T1 or T2 progeny have the same genotypes as those in T0 plants.
[b]Number of base pairs (bp) deleted (-) or inserted (+) into target sites; WT, wild-type. If no percentage is shown, the two genotypes are around 50%:50%.
[c]TIDE only detects indels ≤50 bp.

analysis because morphological defects were observed in one or more mutant lines induced by these gRNAs (**Figure S4**; **Table S4**). A total of 18 possible off-target sites of the 12 gRNAs were identified and off-target mutations were examined in 12 T0 plants, 68 T1 plants and 44 T2 plants by PCR and Sanger sequencing (**Table 3**). No off-target modifications were discovered in the tested plants with or without Cas9, indicating our gRNAs and CRISPR-mediated mutations are highly specific.

Another way to evaluate the specificity of CRISPR/Cas9 is to test the efficiency of gRNAs with a few mismatches against the target sequence in the protospacer. One of our gRNAs targeted two tomato homologs, *Mai5* and *PBL-T1* (bothMai5/PBL-T1; 5'-**g**TAGATCGTAATGGATTGCA-3'; the first nucleotide "C" was converted to "G" to accommodate the transcription initiation requirement of the U6 promoter). The designed 20-bp protospacer sequence exactly matched the target site in *Mai5* but had one mismatch in *PBL-T1* at the third nucleotide from the

5' end (5'-gT**C**GATCGTAATGGATTGCA-3'). We generated five T0 plants that contained the bothMai5/PBL-T1 gRNA construct, all of which had edits in *Mai5* but not in *PBL-T1*, indicating the one mismatch (along with the first nucleotide at the 5' end) in *PBL-T1* appeared to significantly affect Cas9 binding and cleavage activity at the target site. Another gRNA, targeting the tomato *FLS2.2* gene, did not induce targeted modifications in any of the 10 transgenic plants, possibly due to a 1-bp mismatch in the seed region of the gRNA in the Heinz 1706 reference genome (GTCATCAACAT**C**TCGCTTGT) as compared to Rio Grande-PtoR (GTCATCAACAT**T**TCGCTTGT). The reference genome was used for gRNA design and RG-PtoR was used for tomato transformation as it contains the resistance gene *Pto* for investigating *Pto*-mediated immunity in our mutants. This further indicates that CRISPR/Cas9 is highly specific, with even one mismatch in the gRNA rendering the site uncleavable to the Cas9/gRNA complex.

TABLE 3 | Examination of possible off-target mutations caused by 12 selected gRNAs in multiple generations. See also **Tables S4** and **S7**.

gRNAs		Putative off-target locus	Sequence of the putative off-target sites[a]	Predicted by	No. of mis-matches	No. of plants tested	No. of plants with mutations
CathepsinB1-1	#1	SL3.0ch00: 19,581,605 - 19,581,626	gGAATCTGA**C**TTACAA**C**TTATGG	Geneious	2	30 (10 T1 + 20 T2)	0
	#2	SL3.0ch08: 52,560,219 - 52,560,240	g**A**AATCT**T**TCTT**C**CAAGTTAGGG	Geneious	3	20 (10 T1 + 10 T2)	0
ERF5-1	#1	SL3.0ch03: 56,335,989 - 56,336,010 (CDS)	gGTAT**C**GC**A**A**T**GTTCAGAGATGG	Geneious/ CasOFFinder	3	2 (T0)	0
	#2	SL3.0ch03: 56,356,193 - 56,356,214 (CDS)	gGTAT**C**GC**A**A**T**GTTCAGAGATGG	Geneious/ CasOFFinder	3	2 (T0)	0
Fen-1	#1	SL3.0ch11: 22,381,618 - 22,381,638	gATGC-**T**ATAACTTGAGTTAGGG	Geneious	2	17 (10 T1 + 7 T2)	0
Mai5-2	#1	SL3.0ch05: 66,181,295 - 66,181,316 (CDS)	gTCTACGAATATATGCCA**A**TGGG	Geneious/ CasOFFinder	1	1 (T0)	0
Mai5-3	#1[b]	SL3.0ch09: 795,322 - 795,343 (CDS)	gT**C**GATCGTAATGGATTGCAAGG	Geneious/ CasOFFinder	1	3 (1 T0 + 2 T1)	0
	#2	SL3.0ch12: 67,901,371 - 67,901,392 (CDS)	gTA**TC**TC**A**TAATGGATTGCAAGG	Geneious	3	3 (1 T0 + 2 T1)	0
	#3	SL3.0ch12: 67,814,680 - 67,814,701 (CDS)	gTA**TC**TC**A**TAATGGATTGCAAGG	Geneious	3	3 (1 T0 + 2 T1)	0
MKK2-1	#1	SL3.0ch02: 16,695,145 - 16,695,165	gAA**T**ACG-**A**CAGAATCCT**A**GGGG	Geneious	3	17 (2 T0 + 15 T1)	0
MKKK15-1	#1	SL3.0ch02: 48,342,685 - 48,342,706 (intron)	G**C**CCAC-AT**C**GTGTCGACGTGGG	Geneious	3	15 (1 T0 + 7 T1 + 7 T2)	0
PBL-T1-2	#1	SL3.0ch11: 16,102,764 - 16,102,785	gATG**T**CGAG**GC**GGGTCAAATTGG	Geneious	3	4 (1 T0 + 3 T1)	0
RALF2-2	#1	SL3.0ch11: 42,841,387 - 42,841,409	GTTG**A**AG**C**T**T**GGAAGCTCCAAGG	Geneious	3	5 (2 T1 + 3 T2)	0
SOBIR-1	#1	SL3.0ch02: 31,876,612 - 31,876,633	gT**C**TATA**C**A**C**CAGAGCTACCAGG	Geneious	3	11 (4 T1 + 7 T2)	0
TFT10-1	#1	SL3.0ch11: 42,990,626 - 42,990,647 (Intron)	gATTCACTG**A**TAGTA**T**CAGATGG	Geneious/ CasOFFinder	2	7 (1 T0 + 6 T1)	0
	#2	SL3.0ch08: 34,312,506 - 34,312,527 (CDS)	g**C**TTCA**TA**G**T**TAGTAGCAGAAGG	Geneious	4	7 (1 T0 + 6 T1)	0
TFT7-2	#1	SL3.0ch11: 17,271,683 - 17,271,703 (Intron)	gAATGT-AT**G**GGAGCAAGAAAGG	Geneious	2	14 (1 T0 + 13 T1)	0
	#2	SL2.5chr03: 5,874,879- 5,874,902 (CDS)	GAATGT**C**AT**T**GGT**G**CAAGAAGGG	CasOFFinder	3	14 (1 T0 + 13 T1)	0

[a]*The PAM motif occurs at the 3' end of each sequence (AGG, GGG, or TGG). Mismatched bases are in bold and underlined; "g" in lower case means the first nucleotide of the putative off-target sequence is not a "G" but was converted to that nucleotide to accommodate the transcription initiation requirement of the U6 promoter.*
[b]*This is not a true off-target as it was intentionally designed to target a Mai5 homolog PBL-T1).*
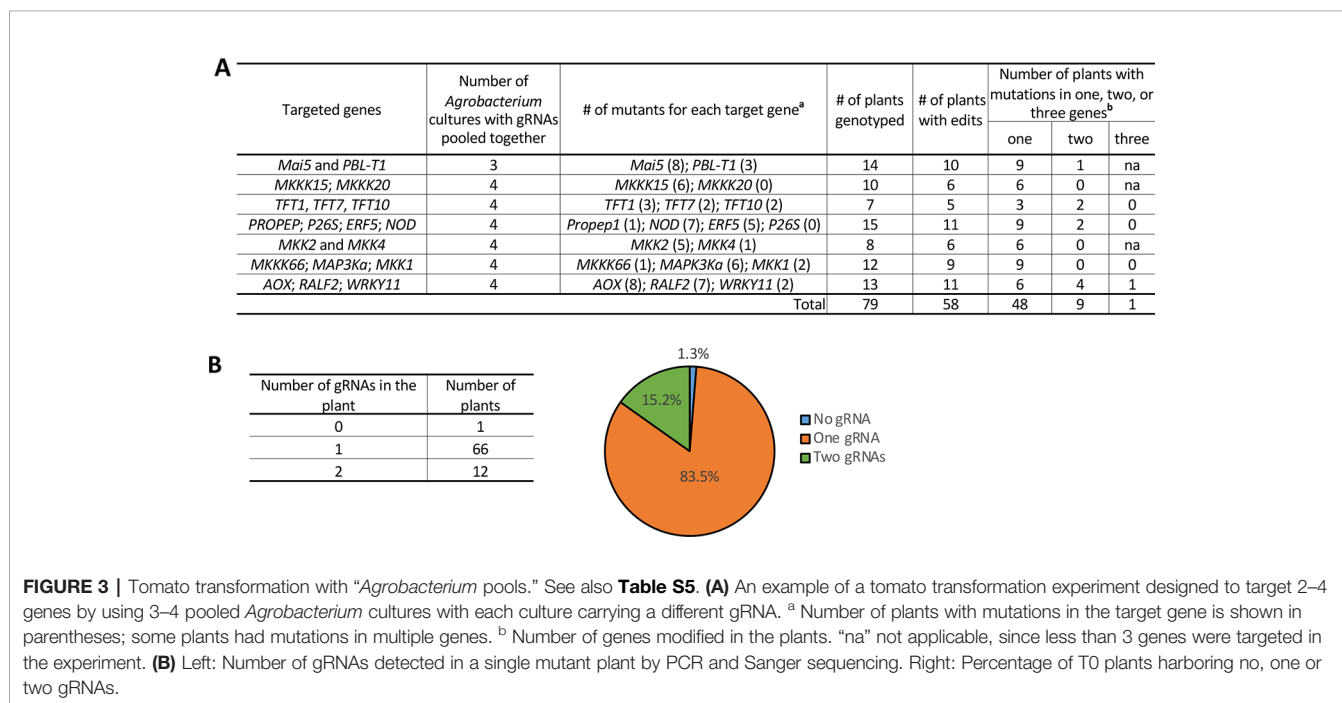
Tomato Transformation With *Agrobacterium* Pools

Tomato transformation is a lengthy and labor-intensive process. In an approach to minimize the number of transformation experiments needed, three to four *Agrobacterium* culture preparations each carrying a different gRNA construct were pooled and used for a single transformation experiment (**Figure 3A**). T0 plants were genotyped for both the presence of T-DNA carrying the gRNAs and for possible mutations in all the targeted genes in the pool. Of the 79 T0 plants generated, 58 plants (73%) contained precise modifications in one or more of the target genes. In terms of the number of target sites edited by CRISPR/Cas9 with pooled gRNAs, 48 (82.8%) of the T0 plants had mutations in just one gene, while 9 plants (15.5%) had mutations in two and 1 (1.7%) had mutations in three genes (**Figure 3A**). Among these T0 plants, 83.5% contained one gRNA cassette and 15.2% contained two different gRNA cassettes, while no plants recovered contained more than two gRNA cassettes integrated into the genome (**Figure 3B**). Interestingly, one mutant plant did not show detectable integration of the T-DNA sequence (expressing *Cas9* and gRNA) but had a mutated gene, suggesting that transient expression of the Cas9/gRNA occurred in this plant. Additionally, we found another type of transient mutation in 8 T0 plants at 10 different target sites (**Table S5**). In these plants, a Cas9/gRNA expression cassette was integrated into the plant genome, as confirmed by PCR and Sanger sequencing, but the gRNA detected was not the one that induced the mutation in the plant (**Table S5**), suggesting that the mutation was caused by another transiently expressed Cas9/gRNA.

DISCUSSION

Our effort to generate a large number of CRISPR/Cas9-induced tomato mutants targeting immunity-associated genes demonstrates that this mutation approach is efficient and robust for gene editing in tomato. Importantly, gene modifications mostly occurred in germline cells and were stably inherited in subsequent generations, similar to those in rice (Zhang et al., 2014) but not as in Arabidopsis, in which most mutations in T0 plants were somatically modified if a strong constitutive promoter was used to regulate the Cas9 expression (Feng et al., 2014; Feng et al., 2018). However, we detected a greater range of deletion and insertion lengths than observed in rice (Zhang et al., 2014), in which only 1-bp insertions and fewer deletion lengths were found, possibly due to different intrinsic DNA repair mechanisms between these two species. These differences could also be due to other factors including different transformation methods or culture conditions, and different sets of target genes that tolerate different degrees of mutations.

Base substitutions induced by the CRISPR/Cas9 system in tomato were very rare in our study. We frequently observed single nucleotide polymorphisms (SNPs) between Rio Grande (used for transformation) and the tomato reference genome (Heinz 1706), but these SNPs were due to natural variation, not mutagenesis, as confirmed by sequencing of the gene regions from untransformed plants. Most of these SNPs were located outside of the protospacer sequence of the gRNA targets, and to date we have only found one gRNA (targeting *Fls2.2*) which had a mismatch in the seed region that inhibited the Cas9 binding and cleavage at the target site. Therefore, it is reasonable to use the tomato reference genome as

Targeted genes	Number of *Agrobacterium* cultures with gRNAs pooled together	# of mutants for each target gene[a]	# of plants genotyped	# of plants with edits	Number of plants with mutations in one, two, or three genes[b]		
					one	two	three
Mai5 and *PBL-T1*	3	*Mai5* (8); *PBL-T1* (3)	14	10	9	1	na
MKKK15; MKKK20	4	*MKKK15* (6); *MKKK20* (0)	10	6	6	0	na
TFT1, TFT7, TFT10	4	*TFT1* (3); *TFT7* (2); *TFT10* (2)	7	5	3	2	0
PROPEP; P26S; ERF5; NOD	4	*Propep1* (1); *NOD* (7); *ERF5* (5); *P26S* (0)	15	11	9	2	0
MKK2 and *MKK4*	4	*MKK2* (5); *MKK4* (1)	8	6	6	0	na
MKKK66; MAP3Ka; MKK1	4	*MKKK66* (1); *MAPK3Ka* (6); *MKK1* (2)	12	9	9	0	0
AOX; RALF2; WRKY11	4	*AOX* (8); *RALF2* (7); *WRKY11* (2)	13	11	6	4	1
		Total	79	58	48	9	1

Number of gRNAs in the plant	Number of plants
0	1
1	66
2	12

FIGURE 3 | Tomato transformation with "*Agrobacterium* pools." See also **Table S5**. **(A)** An example of a tomato transformation experiment designed to target 2–4 genes by using 3–4 pooled *Agrobacterium* cultures with each culture carrying a different gRNA. [a] Number of plants with mutations in the target gene is shown in parentheses; some plants had mutations in multiple genes. [b] Number of genes modified in the plants. "na" not applicable, since less than 3 genes were targeted in the experiment. **(B)** Left: Number of gRNAs detected in a single mutant plant by PCR and Sanger sequencing. Right: Percentage of T0 plants harboring no, one or two gRNAs.

the template for gRNA design and subsequent mutation genotyping in transgenic Rio Grande and likely other tomato cultivars.

Various morphological phenotypes were detected in some mutants compared to wild-type plants. Some of these abnormal phenotypes were associated with all the mutation events occurring in a specific gene, strongly supporting that the mutation itself is responsible for the altered plant growth or development. However, some mutant lines showed unusual morphology associated with certain mutation events but not all, possibly indicating that another off-target mutation occurred in these plants. We therefore investigated a large number of these plants but did not find any evidence of off-target mutations, suggesting other mutations, if they exist, were either caused by tissue culture or *Agrobacterium* transformation, or spontaneous mutations during seed propagation (Tang et al., 2018). Our observations are consistent with previous reports that CRISPR/Cas9 causes few off-target mutations in plants including Arabidopsis (Feng et al., 2014), rice (Zhang et al., 2014; Tang et al., 2018), tomato (Nekrasov et al., 2017), cotton (Li et al., 2019), and maize (Young et al., 2019). True off-targets reported previously in plants showed high sequence homology to the original spacer sequence of gRNAs (Tang et al., 2018), which can be easily avoided by designing highly specific gRNAs using tools such as Geneious and Cas-OFFinder. Based on our data we devised a rule to avoid off-target effects of CRISPR/Cas9 by designing gRNAs whose highest scored potential off-target sites have at least a 1-nt mismatch in the seed sequence or 2-nt mismatches in the full protospacer sequence.

Surprisingly, we found new mutations in the progeny of some T0 plants that did not contain any wild-type allele. These new mutations did not appear to be derived from existing mutations in the T0 plants, as the Cas9-induced modifications were located within the seed sequence of the gRNA protospacer and as little as 1-bp mismatch in the seed sequence can dramatically impair the Cas9 binding and cleavage activity (Jinek et al., 2012). Therefore, we believe the new mutations were derived from chimeric tissue from the T0 plant that was not detected with the one cotyledon/leaf sample we used for mutation genotyping. We are currently advancing lines that have biallelic or heterozygous mutations, or that were chimeric to develop homozygous plants without the presence of Cas9/gRNA sequence. These plants will be used to investigate whether the mutations affect the plant immune response, especially to *P. syringae* pv. tomato.

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DATA AVAILABILITY STATEMENT

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SUPPLEMENTARY MATERIAL

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