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ARTICLE

In silico Prediction and Analysis of Potential Off-Targets and Off-Target Mutation Detection in *StERF3*-Gene Edited Potato Plants

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ABSTRACT

The imperative aspect of the CRISPR/Cas9 system is a short stretch of 20 nucleotides of gRNA that control the overall specificity. Due to the small size, the chance of its multiple occurrences in the genome increases; however, a few mismatches are tolerated by the Cas9 endonuclease activity. An accurate and careful *in silico*-based off-target prediction while target selection is preferred to address the issue. These predictions are based on a comprehensive set of selectable parameters. Therefore, we investigated the possible off-target prediction and their screening in *StERF3* gene-edited potato plants while developing *StERF3*-loss-of-function mutants using CRISPR/Cas9 approach. The 201 off-targets for the selected targets of the *StERF3* gene were predicted, and 79 were filtered as potential off-targets. Of these 79, twenty-five off-targets showed scores with defined cut-off values <0.5 and were analyzed in Sterf3-edited potato plants compared to wild-type plants. No off-targeting was found to have occurred in edited plants.

KEYWORDS

CRISPR/Cas9 system; potato; CCTop algorithm scoring; off-target edits

1 Introduction

Genome editing techniques have grown with the use of engineered biomolecules for directing the modification in the genome. These techniques benefited from the natural DNA repair mechanism after inducing a site-directed break in the double-stranded DNA. Repairing involves a non-templated non-homologous end-joining mechanism or, in the case of break-site homologous templates, a homology-directed repair mechanism [1]. These techniques became increasingly popular, displacing transgenics because of their less toxic effect as well as do not pass through tight regulatory policies. Particularly Zinc-finger nucleases (ZFN), TALE nucleases (TALEN), and CRISPR/Cas systems are used to edit an organism's genome. ZFN and TALEN work as dimers and direct the DNA double-stranded break with the help of the fused non-specific endonuclease domain, FokI [2,3]. There are few reports of successful applications of the ZFN and TALEN in mouse, mammalian, and plant cells [4–6]. However, the limitations of their use include high off-target edits, no ability to target methylated DNA (control several processes, i.e., gene expression, DNA repair, plant growth and development, stress response), and

expensive and tedious designing [7–10]. However, the rapid and widespread adoption of genome editing as a preferred technique to target highly specific changes in genomes is due to the development of the robust CRISPR/Cas9 system [11]. This technique is simple, highly specific, less expensive, and does not need structural validation [12].

CRISPR/Cas9 is a prokaryotes, derived system that produces a double-stranded break at the targeted site of DNA, adjacent to the protospacer adjacent motif (PAM) site (5′-NGG-3′ sequence recognized by Cas9 endonuclease of *Streptococcus pyogenes*). This event is guided by ~20 nucleotide long single guide RNA (sgRNA), and Cas-9 produces the break after protospacer adjacent motif (PAM) recognition. Apart from the on-target site (selected site for targeting), this 20-nucleotide stretch of sgRNA may be found multiple times anywhere in the genome, defined as off-target [13]. However, genotoxicity and the possibility of off-targeting with CRISPR/Cas9 have been documented [14,15]. Off-target editing may have negative consequences like genotoxicity, cytotoxicity, or possible chromosomal rearrangements [16]. Off-target edits can be detected and evaluated to limit undesirable downstream phenotypic consequences. Genome editing for therapeutic purposes in humans strongly focuses on the potential side effects of off-targeting events in the genome. Conversely, crop-specific editing concerns are not directly relevant to mammalian systems [17]. These problems must be considered as they raise safety and regulatory concerns.

Studies based on genome-editing events in crops possibly encounter the most deleterious effect, like off-targeting. The off-targets have been predicted through *in silico* predictors, CHOPCHOP, CRISPOR, Cas-OFFinder, CCTop, Crisflash, etc. [18]. These predictors identify sgRNA and its efficiency for the targeted site and predict possible off-targets. However, different parameters and algorithms of predictors cause inconsistencies in their results. Overall, off-target predictions, criteria are the presence of a defined PAM site (NGG) and the number and position of mismatches. The activity of Cas9 endonuclease strongly correlates with PAM identification and stable gRNA-DNA heteroduplex. The gRNA complements the target site and controls the system's specificity. However, sequences with few mismatches are acceptable by sgRNA/Cas9 and result in off-targeting. The double-strand break may be prevented if the overall sequence of sgRNA has >4 bp [8].

Fewer mismatches and their position upstream to PAM associated with unintended CRISPR/Cas9 editing at untargeted genome loci. Cutting efficiency of the CRISPR/Cas9 system depends on several points, including GC contents of the target site, the presence of the PAM site, and mismatched positions in off-target [19]. The off-target sequences with mismatches ≤4 are more likely to be cut during this event [20]. Moreover, the seed region (8–12 bp upstream from the PAM) with ≤2 mismatches could be at high risk of cleavage [21]. Therefore, a detailed analysis predicting possible off-target and on-target edits should be investigated before CRISPR/Cas-9 targeting. The cleavage based on homology other than the target site results in off-target editing, and the related repercussion is challenging for the researchers [18].

Several *in silico* tools are available to investigate off-target sites. However, the difference among their results has been observed because each follows different criteria and algorithms [22]. The present study aimed to predict possible off-target sites of selected targets of the *StERF3* gene in *Solanum tuberosum*. Our previous study successfully produced *StERF3* full allelic edited potato knockouts (PTP2 and PTP3) [23]. The potential off-target sites of selected targets were identified using the three most commonly used online predictors and were screened in PTP2 and PTP3 plants.

2 Material and Method

2.1 In Silico Analysis of Off-Targets

2.1.1 Off-Target Prediction

The target regions of *the Sterf3* gene for CRISPR/Cas9 editing were selected using CHOPCHOP and CRISPOR web tools. The location of selected targets was inside the *Sterf3* gene. For off-target prediction,

off-target site predictors, CHOPCHOP [24], CRISPOR [25], and CC-TOP [26] were employed. Full description is available at https://link.springer.com/article/10.1007/s11033-022-07958-1.

2.1.2 Putative Off-Target Selection

Off-targets were filtered for further analysis following the criterion of conservation of ≤5 nucleotides upstream to Protospacer adjacent motif (PAM), allowing ≤2 nucleotides as maximum core region (seed region) mismatches [13].

2.1.3 Off-Target Scoring

The off-target scoring was based on the CCTop scoring algorithm and computed using the formula given by Stemmer et al. [26].

$$Score_{off-target} = \sum_{Mismatches} 1.2^{pos*}$$

*pos = position of each mismatch from the 5′ end.

2.1.4 Off-Target Sequence Analysis for Mismatch Distribution

Off-target sequences were analyzed for mismatch distribution magnitude through heat map analysis using TBtools-II (Toolbox for Biologists) v1.112.

2.2 Off-Target Analysis in the Sterf3-Edited Potato Plants

2.2.1 DNA Isolation and PCR Analysis

The detected putative off-target sites were probed in *Sterf3*-edited potato plants to identify off-target events. DNA of fully edited plants (PTP2 &PTP3) and a wild-type plant was isolated using GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific, USA). The PCR amplification was achieved on a thermal cycler, peqSTAR, and products were resolved on 1% agarose gel through gel electrophoresis. The off-target primers for PCR analysis and off-target event identification are in Table S1.

2.2.2 DNA Elution, Cloning, and Sequencing

For each off-target, the DNA band of the required amplicon size was excised from the gel and eluted using Gel Purification Kit (FavorPrep, Taiwan). The eluted product for each off-target was cloned into a cloning vector, pTZ57R/T, using the InsTAclone PCR Cloning Kit (Thermo Scientific, USA) and direct sequenced to Eurofins Genomics DNA Sequencing Services, USA.

2.2.3 Sequence Analysis

The generated sequences were trimmed through BioEdit ver. 7.2.6.1. The alignment of trimmed high-quality sequences of wild type, PTP2, and PTP3 was made using Multiple Alignment using Fast Fourier Transform (MAFFT), a multiple sequence alignment tool.

3 Results and Discussion

Off-targeting is the major concern associated with genome editing executed through the CRISPR-Cas system. Therefore scientists have turned their faces to find the solution regarding off-target prediction while selecting the target sites. *In silico* web-tools are being practiced to address the issue of off-targeting. This study found two hundred and one (201) off-target sequences (Table S2) with canonical PAM for target-1 sgRNA and target-2 sgRNA using the three predictors against the reference *Solanum tuberosum* genome. CHOPCHOP and CCTop recognized fewer off-target sites for target-1 (2 and 20, respectively) and target-2 (17 and 20, respectively). CRISPOR brought the highest number of predicted off-target sites for target-1 and target-2 (48 and 94, respectively). After filtering for sequences with ≤5 mismatches, 79 sequences (Table 1) were obtained as potential off-targets for both target sites (Fig. 1). The potential off-targets with >4 mismatches prevent double-stranded break induction [13].

Table 1: Potential off-targets of target-1 and target-2 with their CCTop algorithm score

ID*	Predictors	Sequence	MM**	Genomic location	Off-target score
2	**CHOPCHOP**	tCTaTcAGATTTCCGATCTC CGG	3	CP046691.1:9647674-9647695	0.75
74		AACAGAGGAgCaAAAAACAG AGG	2	CP055244.1:13377863-13377885	0.12
83		AACAtAGGAgCaAAAAACAG TGG	3	CP023767.1:9582767-9582789	0.31
84		AACAGAGGAgggAAAAACAG AGG	3	CP055240.1:38608045-38608067	0.18
85		AgCAGAGGAggCAAAAACAG AGG	3	CP046684.1:16274350-16274372	0.45
20	**CRISPOR**	tCaGaTAGAcTTCCGATCTC AGG	4	CM020968.1:37796790-37796812	0.90
21		cCTtTgAGATTTCCaATCTC TGG	4	CM020971.1:32452975-32452997	0.78
22		ctaGTTAaATTTCCGATCTC AAG	4	CM020970.1:4525194-4525216	1.07
31		tgTGTTAGtTTTCCcATCTC CGG	4	CM020966.1:24450346-24450368	0.82
88		AACAGnGGAACCAAAAACAG GGG	1	CM020976.1:47243234-47243256	0.15
95		AACAGAGGAtggAAAAACAG AGG	3	CM020969.1:57556751-57556773	0.18
97		AACAGAGGAnggAAAAACAG AGG	3	CM020967.1:54094652-54094674	0.18
98		AAagGAaGAACCAAAAACAG AGG	3	CM020975.1:50969351-50969373	0.61
100		AACAGAGGAgaaAAAAACAG AGG	3	CM020976.1:41234526-41234548	0.18
101		AACAGAGGAgaaAAAAACAG AGG	3	CM020976.1:34814665-34814687	0.18
103		AgCAGAacAACCAAAAACAG AAG	3	CM020968.1:59517140-59517162	0.55
104		AACAGAGGAggaAAAAACAG AGG	3	CM020974.1:10791171-10791193	0.18
107		AtCAGAaGAAgCAnAAACAG AGG	3	CM020966.1:248999-249021	0.54
108		AAaAtAGGAACCtAAAACAG AAG	3	CM020969.1:41630272-41630294	0.49
109		AAaAtAGGAACCtAAAACAG AAG	3	CM020969.1:42660028-42660050	0.49
117		AgaAGAGGAACaAtAAACAG TGG	4	CM020976.1:51350309-51350331	0.67
118		AgCAGAGGAgaaAAAAACAG GGG	4	CM020974.1:42397568-42397590	0.51
119		AACAGAGGAgaagAAAACAG AGG	4	CM020974.1:46256017-46256039	0.23
120		cACtGAGaAAtCAAAAACAG AGG	4	CM020965.1:90980754-90980776	0.77
121		cACtGAGaAAtCAAAAACAG AGG	4	CM020965.1:42454779-42454801	0.77
126		AAaAcAGGAAaCAAcAACAG TGG	4	CM020973.1:36470626-36470648	0.54
127		gACAGAGaAAgtAAAAACAG CGG	4	CM020965.1:36617399-36617421	0.60
128		gACAGAGaAAgtAAAAACAG CGG	4	CM020965.1:85143374-85143396	0.60
134		AACAGAGtcAtaAAAAACAG GGG	4	CM020973.1:1324107-1324129	0.30
139		AAaAGAGGAgggAAAAACAG AGG	4	CM020970.1:11775545-11775567	0.45
140		AAaAGAGGAAggAgAAACAG AGG	4	CM020972.1:602799-602821	0.41
154		tAgAGAGGAACaAtAAACAG GGG	4	CM020974.1:5838378-5838400	0.73
155		AACAaAGGAAtagAAAACAG AGG	4	CM020965.1:84700490-84700512	0.34
156		AACAaAGGAAtagAAAACAG AGG	4	CM020965.1:36174515-36174537	0.34
158		AcCAGAGgGagAAAAAACAG GGG	4	CM020974.1:41145770-41145792	0.56
163		AgCAGAGGAgggAAAAACAG AGG	4	CM020970.1:16244543-16244565	0.50
164		AACAGgGGAgggAAAAACAG AGG	4	CM020967.1:53841207-53841229	0.34
170		AAaAnAGGAAaCAAcAACAG TGG	4	CM020967.1:24837159-24837181	0.54
171		AgCtGAaGAACaAAnAACAG TGG	5	CM020970.1:9794546-9794568	0.75
175		AngAGAtGAACaAgAAACAG GGG	5	CM020967.1:7697332-7697354	0.80
178		AgCAGgaGAAaCAAnAACAG GGG	5	CM020972.1:49869264-49869286	0.69
180		AACAGtGagAnaAAAAACAG AGG	5	CM020969.1:21341798-21341820	0.46

(Continued)

Table 1 (continued)

ID*	Predictors	Sequence	MM**	Genomic location	Off-target score
51		ACgGTgAGATTTtCGATCTC AGG	3	PGSC0003DMG400043120:41011715-41011737	0.46
52		tCaGaTAGAcTTCCGATCTC AGG	4	PGSC0003DMG400024116:37826925-37826947	0.91
53		cCTtTgAGATTTCCaATCTC TGG	4	PGSC0003DMG400012992:32485873-32485895	0.78
54		AtTGcTgGATTTCCcATCTC AGG	4	PGSC0003DMG400025640:7255545-7255567	0.66
55		tgTGTTAGtTTTCCcATCTC CGG	4	PGSC0003DMG4000376152450128 2-24501304	0.82
56		gtgGTaAtATTTCCGATCTC CGG	5	PGSC0003DMG400016685:5762012-5762034	1.23
57		cCTtagAcATTTCCGATCTC TGG	5	PGSC0003DMG400043680:63165920-63165942	1.05
58		ttaGTgAGATTaCCGATCTC AGG	5	PGSC0003DMG400005538:60573098-60573120	1.17
59		caaGTTAGgTgTCCGATCTC CGG	5	ChrUn:750262-750284	1.12
60		caaGTTAGgTgTCCGATCTC CGG	5	PGSC0003DMG400010194:32029134-32029156	1.12
61		caaGTTAGgTgTCCGATCTC CGG	5	PGSC0003DMG400033319:43835082-43835104	1.12
62		caaGTTAGAagTCCGATCTC CGG	5	PGSC0003DMG400005359:30574812-30574834	1.10
63		AtaGaTcGATTcCCGATCTC CGG	5	PGSC0003DMG400043483:28169975-28169997	0.95
64		tCTtcTAcATTcCCGATCTC AGG	5	PGSC0003DMG400040798:7317094-7317116	0.94
65		tCTtcTAcATTcCCGATCTC AGG	5	PGSC0003DMG400021273:2180472-2180494	0.94
66		AtTGggAtATTTtCGATCTC TGG	5	PGSC0003DMG400031149:61689544-61689566	0.81
67		AtTGggAtATTTtCGATCTC TGG	5	PGSC0003DMG400031149:61688249-61688271	0.80
68		ttTGTgAtATTTCaGATCTC AGG	5	PGSC0003DMG400030763: 40835419-40835441	0.99
182	**CC-TOP**	AACAGAGGAgCaAAAAACAG AGG	2	CP046684.1:12078296-12078318	0.12
183		AACAGAGGAgCaAAAAACAG AGG	2	CP046691.1:12078413-12078435	0.12
184		AACAGAGGAgCaAAAAACAG AGG	2	PGSC0003DMG400040936:12078716-12078738	0.12
185		AACAGAGGAAggAAAAACAG AGG	2	PGSC0003DMG400019229:54194552-54194574	0.11
186		AAagGAaGAACCAAAAACAG AGG	3	PGSC0003DMG400003836:51031649-51031671	0.61
187		AAaAtAtGAACtAAAAACAG GGG	4	PGSC0003DMG40004212:40929783-40929805	0.63
188		cAtAtAatAACCAAAAACAG TGG	5	PGSC0003DMG400009223:8693438-8693460	1.06
189		tAttGAaGcACCAAAAACAG AGG	5	ChrUn:62279993-62280015	1.08
190		tAttGAaGcACCAAAAACAG AGG	5	PGSC0003DMG400015054:13442315-13442337	1.08
191		tAttGAaGcACCAAAAACAG AGG	5	PGSC0003DMG400037631:39172783-39172805	1.08
193		gACtGtaGcACCAAAAACAG AGG	5	PGSC0003DMG400019465:5179353-5179375	0.97
194		tAgAcAaGAcCCAAAAACAG GGG	5	PGSC0003DMG400044838:22339447-22339469	1.04
195		AtgtGgGGAgCAAAAACAG GGG	5	PGSC0003DMG40002451:54665817-54665839	1.02
196		AtgcGAtGAACaAAAAACAG GGG	5	PGSC0003DMG400010348:27800425-27800447	0.98
197		AAgcaAGaAAaCAAAAACAG GGG	5	PGSC0003DMG400021062:24035334-24035356	0.84
198		AAaAcgaGAAgCAAAAACAG AGG	5	PGSC0003DMG400008144:58555647-58555669	0.79
199		tAgAGAGtgtCCAAAAACAG TGG	5	PGSC0003DMG400013011:38463720-38463742	0.92
200		AtgAGctGAACaAAAAACAG GGG	5	ChrUn:97090264-97090286	0.91
201		tAttGAaGcACCAAAAACAG AGG	5	PGSC0003DMG400037631:39174901-39174923	1.08

Note: *ID number is given in Table S2, **Mismatches.

The seed region determines the overall target specificity. The closer the mismatches to PAM, the more chances to abolish double-strand break [13]. Therefore, we selected the off-targets with ≤2 bp mismatches in the seed region because Cas9 can tolerate mismatches up to 5 and does more off-target edits [13,27,28]. Conserving a few base pairs adjacent to PAM is also important [21,29]. Hence, we selected off-target sequences following 5 bp conservation. Similarly, Carneiro et al. [22] also used the criteria of 5 bp conservation upstream to PAM.

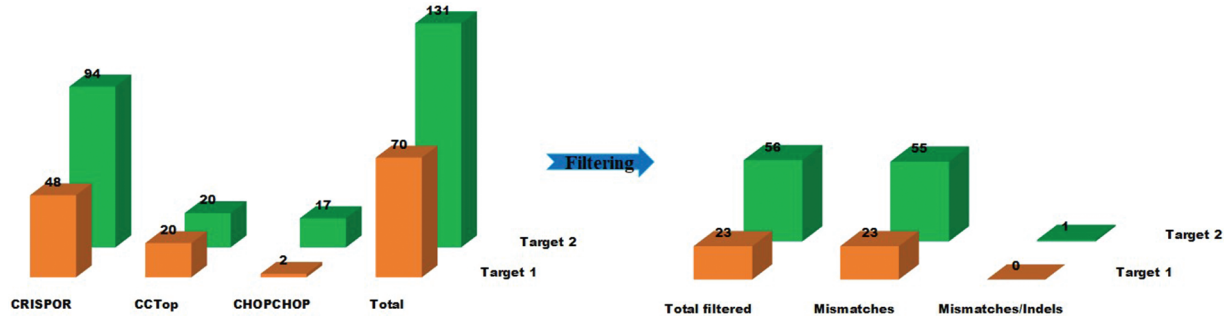

Figure 1: Off-target prediction to target-1 and target-2 of the *Sterf3* gene by employing off-target site predictors, CHOPCHOP, CRISPOR, and CC-TOP and filter criteria. Graphically illustrate the off-target predictions of both targets before and after filtering steps. The illustration shows the number of sequences returned for each of the three predictors and the number of sequences after the filtering step

In off-target sequences with mismatches only, PAM is located at positions 21–23, while in off-target sequences with mismatches and indels, PAM location was found at positions 20 to 22. The non-random distribution was observed, as most of these mismatches were concentrated at positions 1–12 in off-target sequences (Fig. 2). Fifteen identical off-target sequences were observed more than once in different genomic positions, which could be responsible for this distribution.

The cleavage probability for putative off-targets was scored based on the CCTop score algorithm. The higher the CCTop score, the lower the chance of off-targeting [30]. This scoring method is based on the position of mismatched nucleotides from the PAM [26]. Here in this study, we defined 0.5 as a cut-off value, and the off-targets with a score of less than 0.5 were selected for identifying off-target events in fully edited plants of *Solanum tuberosum*. Of these 79 off-targets, twenty-five off-targets with a score <0.5 were selected for off-targeting analysis of *Sterf3*-edited potato plants.

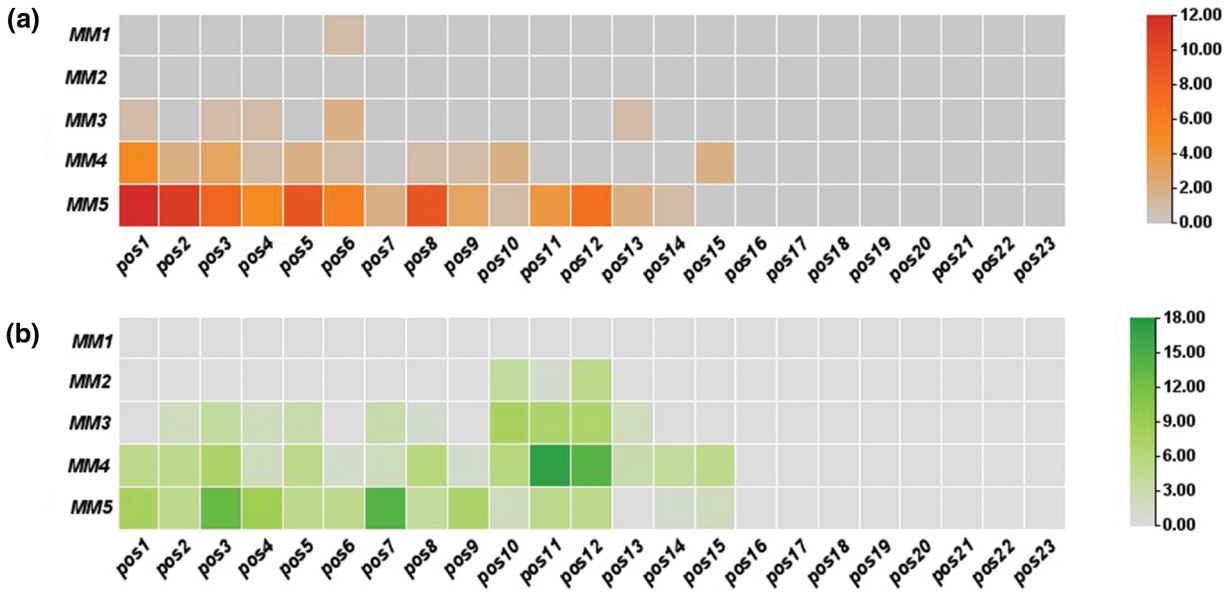

Figure 2: (Continued)

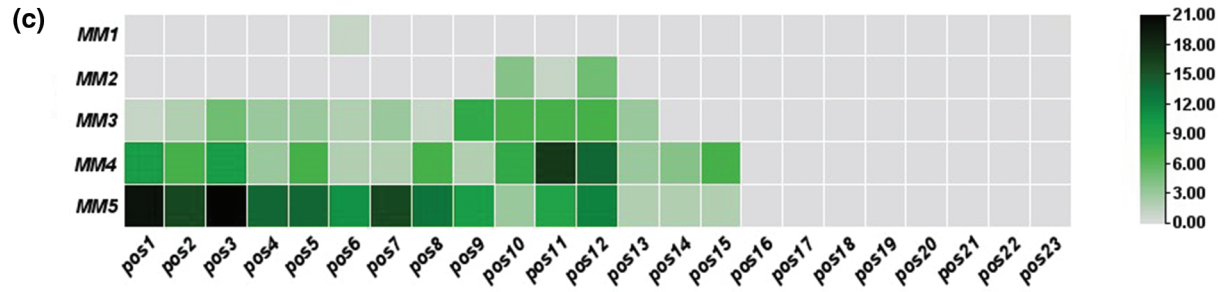

Figure 2: Distribution magnitude and frequency of mismatches in predicted off-targets by heat map analysis; (a) distribution magnitude of mismatches in off-targets of target-1; (b) distribution magnitude of mismatches in off-targets of target-2; and (c) distribution magnitude of mismatches in off-targets of both targets

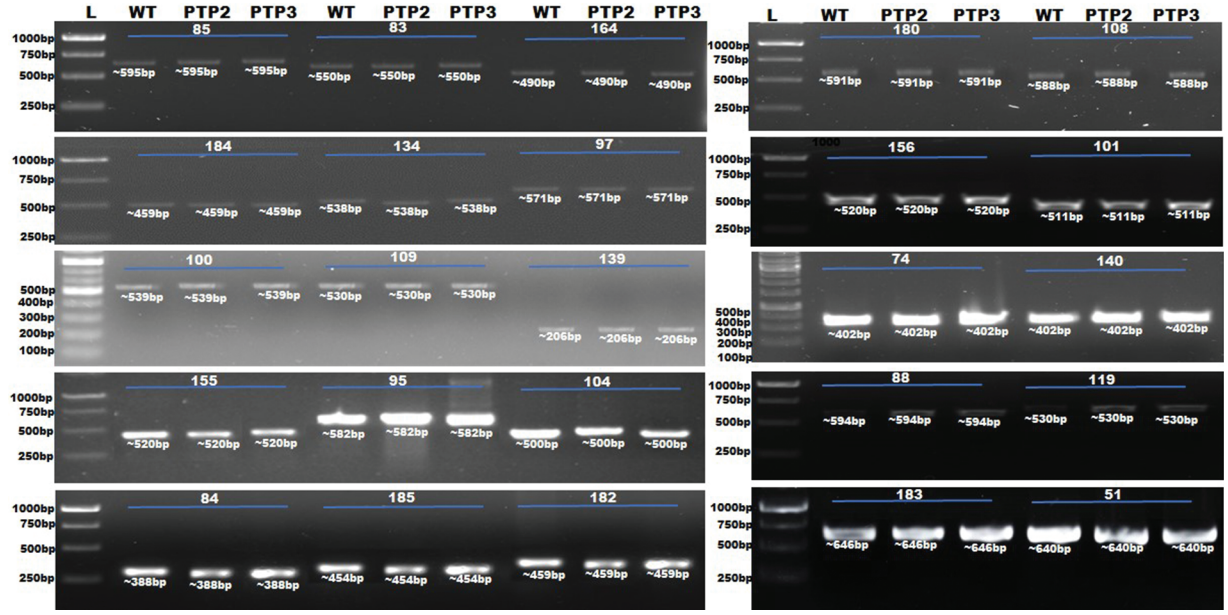

Figure 3: PCR amplification of off-targets in *Sterf3*-edited potato plants (PTP2 and PTP3) compared to wild-type plants (WT). ***L** = 1Kb DNA ladder **51** = PGSC0003DMG400043120:41011715-41011737, **74** = CP055244.1:13377863-13377885, **83** = CP023767.1:9582767-9582789, **84** = CP055240.1 :38608045-38608067, **85** = CP046684.1: 16274350-16274372, **184** = PGSC0003DMG400040936: 12078716-12078738, **88** = CM020976.1 47.24 Mbp_CM020976.1_47243233, **95** = CM020969.1 57.56 Mbp_CM020969.1_57556750, **97** = CM020967.1 54.09 Mbp_CM020967.1_54094651, **100** = CM020976.1 41.23 Mbp_CM020976.1_41234525, **101** = CM020976.1 34.81 Mbp_ CM020976.1_34814664, **104** = CM020974.1 10.79 Mbp_CM020974.1_10791170, **109** = CM020969.1 42.66 Mbp_CM020969.1_42660027, **108** = CM020969.1 41.63 Mbp_CM020969.1_41630271, **119** = CM020974.1 46.26 Mbp_CM020974.1_46256016, **134** = CM020973.1 1.32 Mbp_ CM020973.1_1324106, **139** = CM020970.1 11.78 Mbp_CM020970.1_11775544, **140** = CM020972.1 602.80 Kbp_CM020972.1_602798, **155** = CM020965.1 84.70 Mbp_CM020965.1_84700489, **156** = CM020965.1 36.17 Mbp_CM020965.1_36174514, **164** = CM020967.1 53.84 Mbp_ CM020967.1_53841206, **180** = CM020969.121.34 Mbp_CM020969.1_21341797, **182** = CP046684. 1:12078296-12078318, **183** = CP046691.1:12078413-12078435, **185** = PGSC0003DMG4000 19229:54194552-54194574

Doench et al.'s [30] work for minimizing off-target effects of CRISPR-Cas9 documented that criteria for cut-off value should be based on experimental observation. Carneiro et al. [22] followed Doench et al. [30] and set the cut-off value greater than 0.2. So, for more stringency, we set the cut-off value <0.5 and screened the material for more off-targets. As the cut-off value is high, more off-targets would be selected for analysis to minimize the off-target effects of CRISPR-Cas9 in the edited plants.

3.1 Detection of Off-Target Mutations in Edited Potato Plants

The putative off-target sites of selected targeted sites of the *StERF3* gene were probed for off-target events in *Sterf3*-edited potato plants. DNA fragments with putative off-target sites amplified through PCR amplification (Fig. 3) were cloned in a TA cloning vector for sequencing. The primers to amplify off-target sites were selected following the criteria, 600 bp-sanger reads. Sequencing data of PCR amplicons were aligned, which showed no mutation at the putative off-target sites of *StERF3* knockout mutants (Supplementary File).

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Author Contributions: The authors confirm their contribution to the paper as follows: Study conception and design: Siddra Ijaz and Imran Ul Haq; Data collection: Hafiza Arooj Razzaq, Siddra Ijaz, and Imran Ul Haq; Analysis and interpretation of results: Siddra Ijaz, Imran Ul Haq, and Hafiza Arooj Razzaq; Draft manuscript preparation: Hafiza Arooj Razzaq, Siddra Ijaz, Imran Ul Haq, and Faisal Saeed Awan. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

Table S1: List of primers use to amplify twenty-five putative off-targets of two targets (target-1 & target-2)

Table S2: Off-target sites prediction of two targets with three online predictors

Supplementary File: Alignment