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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

CRISPR/Cas9 mediated genome-editing of the *eIF4E* gene in Tomato

토마토에서 CRISPR/Cas9 매개의 *eIF4E* 유전자교정

FEBRUARY, 2020

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ABSTRACT

The development of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology has made targeted mutations possible in virtually any organisms. Eukaryotic translation initiation factor 4E (eIF4E) is the protein encoded by recessive virus resistance genes in plants. In this study, the *eIF4E* gene was chosen as a target for genome editing (GE) in *Solanum lycopersicum* cv. Micro-Tom. The *Cas9* gene together with gRNA for *phytoene desaturase* (*PDS*) or *eIF4E* genes were delivered to Micro-Tom via *Agrobacterium*-mediated method and transgenic tomato plants carrying *Cas9* and gRNA were obtained. The photo bleaching phenotype was observed in four out of 113 explants targeted for *PDS* demonstrating homozygous GE plants can be obtained in T_0 generation. To obtain the *eIF4E* edited tomato plants, transgenic plants were analyzed by sequencing the *eIF4E* gene of

transgenic plants. A total of 16 *eIF4E* edited E_0 plants containing various sizes of deletion ranging from 11 to 43 bp in *eIF4E1* were obtained. E_0 plants were self-pollinated and five homozygous E_1 mutant lines for *eIF4E1* were obtained. *Tobacco etch virus* (TEV) was inoculated to E_2 progeny. No resistance to TEV was observed in two *eIF4E1* mutants, which may be due to the redundancy of the *eIF4E* gene. This study demonstrates that CRISPR/Cas9 technology can be used as a useful tool for mutating to desired targets.

Ke**ywords**: Tomato, CRISPR/Cas9, genome editing, Eukaryotic translation initiation factor 4E (*eIF4E)*, Potyvirus, Phytoene desaturase (*PDS*)

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LIST OF ABBREVIATIONS

CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/ CRISPR associated protein-9
Dpi	Day post inoculation
DSB	Double strand break
eIF4E	Eukaryotic translation initiation factor 4E
GE	Genome editing
gRNA	Guide RNA
HDR	Homology directed repair
Hpt	Hypoxanthine phosphoribosyltransferase
NHEJ	Non-homologous end joining
PAM	Protospacer adjacent motif
PDS	Phytoene desaturase
TALEN	Transcription activator-like effector nuclease
TILLING	Targeted local lesions in generation
VPg	Viral genome-linked protein
ZFN	Zinc finger nucleases

INTRODUCTION

Tomato (*Solanum lycopersicum*) is a plant belonging to the Solanaceae family and are one of the most widely cultivated crops in the world and of great agricultural value. It is also actively studied in many areas such as fruit color (Liu et al, 2015), disease resistance (Lapidot, 2015) and ripening process (Vrebalov, 2002) as good model organisms in molecular biology.

Plant disease is one of the major factors affecting crop production worldwide. The damage caused by the virus has been on the rise lately. Methods such as pesticides and physical control may be used to control plant diseases, but they are not sustainable and effective. Therefore, the most effective way to control the current virus is to develop virus-resistant varieties (Nicaise, 2014).

Potyvirus accounts for about 30% of existing plant viruses and is known to cause enormous damages to plants (Ward and Shukla, 1991). Potyvirus has a single-stranded positive RNA combined with viral genome-linked protein (VPg) and poly(A) tail at 5'- and 3'-ends, respectively. Approximately 10 kb monopartite genome encodes single polyprotein which is truncated by proteases to multiple functional proteins (Wylie et al., 2017).

Plant viruses are simple structured obligate intracellular parasites with minimal genes and rely on host machineries for the life cycle and systemic movement of the virus (Nelson and Citovski, 2005). Eukaryotic organisms have eukaryotic translation

initialization factors (eIF). Eukaryotic translation initiation factor 4E (*eIF4E*) is one components of the multi-complex protein involved in the process of mRNA translation, and starts protein translation by enabling recognition and interaction with the cap structure of cellular mRNA. Viral genome-linked protein (VPg) of the potyvirus is covalently connected to the 5' end of viral RNA and can replace the role of the cap structure in mRNA during protein translation. In the previous studies, it was reported that the physical interaction between the host factor *eIF4E* (or its homolog (iso)4E) and VPg is very important for the infection of several species of potyviruses (Wittmann et al., 1997; Leonard et al., 2000; Grzela et al., 2006; Miyoshi et al., 2006; Robaglia and Caranta, 2006). It has also been reported that mutations that occur in these host factors inhibit the interaction between the host factor and the VPg and consequently prevent infection of the virus (Duprat et al., 2002; Lellis et al., 2002; Rodríguez-Hernández et al., 2012; Sato et al., 2005). There have been many reports to develop virus resistant plants by silencing host factors to interrupt the interaction of host factors with VPg. Recently, broad virus resistance were reported in tomato and melon by silencing the *eIF4E* gene (Mazier et al., 2011; Rodríguez-Hernández et al., 2012). In addition, knockout allele of *eIF4E*, which gives narrow spectrum virus resistance, by TILLING (targeted local lesions in generation) was reported (Piron et al., 2010).

CRISPR/Cas9 (Clustered Regularly Interspaced Palindromic Repeats/CRISPR-associated protein9) is one of the genome editing (GE) techniques derived from the immune system against virus infection in *S. pyogenes*.

CRISPR/Cas9 has been utilized in site-directed mutations various organisms including microorganism, animal, human cells, and plants (Shapiro et al., 2018; Wu et al., 2013; Zhang et al., 2017; Liang et al., 2015). Since its first report in 2012, the studies on GE have been exploded due to its easiness, low cost and significantly reduced time for construct preparation compared to conventional GE tools such as ZFN and TALEN (Cong and Zhang., 2015). CRISPR/Cas9 causes DNA double-stranded break (DSB) at the target site when a guide RNA combines with 20-nucleotide DNA target sequence located at 5' protospacer adjacent motif (PAM) (Jinek et al., 2012). This generally induces the in-cell repair work non-homologus end joining (NHEJ) pathway resulting in random mutations such as substitution or InDels, which can change the open reading frame of the protein, or cause premature stop codon (Lieber., 2010).

Similar to TILLING, which has been widely used in mutation studies, CRISPR/Cas9 is to obtain mutations, but different in that it induces mutations in specific sites. (McCallum et al., 2000). The cost and time of TILLING is higher and longer compared to CRISPR/Cas9, while off-target effects can cause unwanted mutations in CRISPR/Cas9. Recently, experiments inducing potyvirus resistance by CRIPSR/Cas9 genome editing have been reported in plants. For instance, the *eIFiso4E* gene was knocked out for Turnip mosaic virus (TuMV) resistance and they could generate transgene-free mutants (Pyott et al., 2016). In a similar manner, the *eIF4E* gene was targeted to knock-out and broad spectrum virus resistances were reported for *Cucumber vein yellowing virus* (CVYV), *Zucchini yellow mosaic virus*

(ZYMV), *Papaya ringspot mosaic virus*-w (PRSV-W) (Chandrasekaran et al., 2016).

In this study, the *eIF4E* gene was mutated in tomato using CRISPR/Cas9 technology via *Agrobacterium*-mediated transformation. E_0 transgenic plants containing *eIF4E* mutations were advanced to E_2 to obtain homozygous lines. Subsequently, virus resistance of mutant lines was tested for *Tobacco etch virus* (TEV).

LITERATURE REVIEW

1. CRISPR/Cas9

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system is a genome editing technique that can cause artificial mutation in the sequence of desired areas. CRISPR/Cas systems are derived from the bacterial adaptive immune system that exists to counter genetic material such as externally invasive bacteriophage or plasmid (Wiedenheft et al., 2012). In order for the system to work, two components are required : a guide RNA (gRNA) which can complementary match with target region in DNA, a Cas9 nuclease which can result double-stranded break (DSB). Forming Cas9-gRNA complex, the complex recognizes 20-nucleotides DNA sequence which is in the 5' region of protospacer adjacent motif (PAM; NGG in the case of SpCas9) and induce double-stranded break (DSB). The damaged DNA is subsequently repaired by cellular mechanism which is divided into two ways. One is error-prone non-homologous end joining (NHEJ) pathway. It activates when donor template absences and causes random Insertions and deletions (InDels) or substitutions at the DSB site. Another is error-free homology directed repair (HDR) pathway. It activates when donor templated presences and creates desired mutations through homology recombination (Jiang and Doudna., 2017). In general, plants prevalently utilize NHEJ mechanisms for DSB repair (Puchta., 2004). This

mechanism function during the overall cell cycle (except for mitosis), whereas HDR acts only in the late S and G2 phases (Orthwein et al., 2015).

OFF-target effect

Off-target effect, non-specific and unintended genetic modifications caused by genome editing, is considered one of the major obstacles of the CRISPR/Cas9 system in human cells (Fu et al., 2013; Hsu et al., 2013). To overcome this issue, various attempts like double nickase (Ran et al., 2013), dCas9-FokI fusions (Guilinger et al., 2014; Tsai et al., 2014), high fidelity Cas9 versions, such as SpCas9-HF (Kleinstiver et al., 2016; Slaymaker et al., 2016) for increasing target specificity have been tried. However for plants, off-target issue can weigh less than when applied to humans. This is because mutations caused by off-target can be easily separated through segmentation or removed by backcrossing, and can be a clear advantage when compared to the mammalian system (Hahn and Nekrasov., 2018). So far, it has been reported that CRISPR/Cas can mutate into off-target sites that are very similar to the target (Lawrenson et al., 2015; Zhang et al., 2018; Tang et al., 2018). However, analysis of the CRISPR/Cas off-target with genome wide scale is still limited to some plants such as rice, corn, tomato, and Arabidopsis (Feng et al., 2014, 2018; Peterson et al., 2016; Nekrasov et al., 2017; Lee et al. 2018; Tang et al. 2018). In Tang et al experiment, Two types of CRISPR system,SpCas9-sgRNAs and Cas12a-CRISPR RNAas(crRNAs) to multiple gene targets were used in rice. They estimated off-targeting rates by whole genome

sequencing (WGS). Most of the mutations they detected in CRISPR/Cas-expressing lines or their progeny, were background mutations caused during seed amplification or tissue culture. Only one out of the twelve Cas9 sgRNAs resulted in off-target mutations in a number of loci that shared significant similarity with the targeted locus, In the case of Cas12a, no off-target mutations were detected. only a limited number of Cas9 sgRNAs and Cas12a crRNAs were tested in this study, its results are consistent with other recent reports showing that CRISPR/Cas is highly precise when applied in plants (Nekrasov et al. 2013, 2017; Peterson et al. 2016; Feng et al. 2018; Lee et al. 2018).

2. Recessive resistance

Recessive resistance is a disease resistance that occurs when a pathogen lacks the instrumental gene it needs to survive, or when interaction is inhibited. Up to now, half of the genes associated with viral resistance in the crop are known as recessive resistance genes (Kang et al., 2005). In general, recessive resistance acts more strongly than dominant resistance, and is somewhat broad in particular, with the characteristic that the resistance does not easily collapse by mutation generation. (Hashimoto et al., 2016; Truniger and Aranda., 2009; Wang and Krishnaswamy., 2012).

Although plant viruses still encode a number of essential proteins like coat proteins, movement proteins, replication enzymes, their coding capacity is limited

and they must rely on host factors in every steps of the infection cycle (Wang et al., 2015). Viruses do not normally encode canonical translation factors, but have developed a wide array of strategies to highjack translation factors from their hosts and favor the translation of viral RNAs to the detriment of endogenous mRNAs. Majority of naturally occurring plant recessive resistance genes have been mapped to mutations in isoforms of translation initiation factors eIF4E and eIF4G. These mutations generally hinder the interactions of host factors with viral RNAs or proteins (Wang and Krishnaswamy., 2012; Le Gall et al., 2011).

In eukaryotes, mRNA translation is predominantly mediated by the 5' 7-methyl-guanosine cap and 3' poly(A) tail via a cap-dependent mechanism. Cap-dependent translation initiation is a multistep process requiring the assembly of a super mRNA-protein complex by different eIFs, including the eIF4F complex (eIF4E, eIF4G, and eIF4A).

The exclusivity of different potyviral VPgs for their cognate eIF partners has formed the basis for natural, recessive resistance to a wide range of potyviruses in various crops. For example, pepper *pvr2* (Ruffel et al., 2002), lettuce *mo1* (Nicaise et al., 2003), pea *sbm1* (Gao et al., 2004), tomato *pot-1* (Ruffel et al., 2005), barley *rym4* (Kanyuka et al., 2005; Stein et al., 2005), melon *nsv* (Nieto et al., 2006) bean *bc-3* (Naderpour et al., 2010) and potato *eva1* (Duan et al., 2012) have all been mapped to either *eIF4E* or *eIF(iso)4E* homologues.

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MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of the *Solanum lycopersicum* 'Micro-Tom' were surface sterilized in 70% ethanol for 1 min and in 2% NaOCl with one drop Tween 20 for 15 min, and then rinsed four-five times with sterilized water until chlorine smell disappeared. The seeds were germinated on 1/2 MS medium (Murashige and Skoog 1962) contained 20 g/L Sucrose, 8 g/L plant agar. The pH was adjusted to 5.8 ± 1 adjusted with HCl or NaOH and autoclaved at 121℃ for 20 min. The medium sowed seeds was wrapped with aluminum foil to make dark conditions for 3 days, and then removed to promote uniform germination in light conditions. All the culture were grown at 24℃ under 16 h light/8 h dark cycle under the fluorescent light. Cotyledons of 6 to 7 day-old seedlings before the first true leaves emerged were used as explants for tissue culture. Rooted plants were transferred to soil to harden. Hardened plants were grown in a 24℃ growth room.

Guide RNA design and vector construction

The target sequence of the gRNA was designed by using CCTop-CRISPR/Cas9 target online predictor (https://crispr.cos.uni-heidelberg.de/index.html). I used the pHSE401 vector containing the maize codon optimized the *Cas9* gene. The 23 bp sequence of corresponding primers,

PDSgRNA_F and PDSgRNA_R (or eIF4E1gRNA_F and eIF4E1gRNA_R) flanked by *Bsa*I site, were annealed and cloned into *Bsa*I digested entry pHSE401 vector by golden-gate cloning according to the previously reported method (Xing et al., 2014).

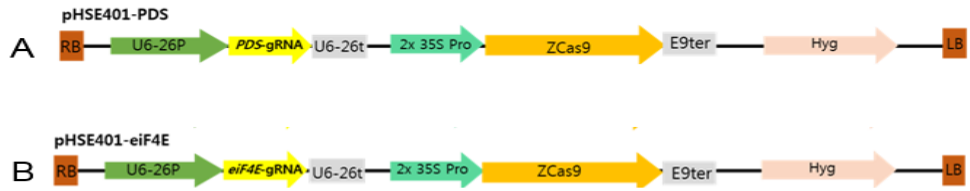

Figure 1. Schematic diagrams of the CRISPR/Cas9 vector constructs. (A) Schematic diagram of the cassette expressing the maize codon optimized the *Cas9* gene under the 35S promoter and the PDS-gRNA sequence driven by the Arabidopsis U6-26 pol III promoter (pHSE401-PDS) (B) Schematic diagram of the cassette expressing the maize codon optimized the *Cas9* gene under the 35S promoter and the eIF4E-gRNA sequence driven by the Arabidopsis U6-26 pol III promoter (pHSE401-eIF4E).

Table 1. Primers for CRISPR/Cas9 vector construction

Primer	Sequence (5’ to 3’)	Tm (℃)	GC contents (%)
PDSgRNA_F	ATTGCTGTTAACTTGAGAGTCCA	54.9	39.13
PDSgRNA_R	AAACTGGACTCTCAAGTTAACAG	54.9	39.13
eIF4EgRNA_F	ATTGAGTTGAAGGCCGCCGATGG	62.0	56.52
eIF4EgRNA_R	AAACCCATCGGCGGCCTTCAACT	62.0	56.52

Agrobacterium culture for transformation

CRISPR/Cas9 vectors were transformed by electroporation into the *Agrobacterium tumefaciens* strain GV3101 for stable transformation. A single colony from the selection medium was sequenced to confirm the sequence of the construct. Confirmed single colony was inoculated to 15 ml of liquid LB medium that contained 50 μg/mL kanamycin and 50 μg/mL rifampicin and incubated in a shaking incubator at 28℃ for 12-16 hr. The *Agrobacterium* suspension was centrifuged at 8000 rpm for 10 min at 20℃ to collect pellet. The pellet was completely resuspended in 1/2 liquid MS medium containing 3% sucrose with 200μM acetosyringone and adjusted OD_{600} of 0.6.

Agrobacterium-mediated tomato transformation

Cotyledon explants were cut off in sterile conditions, the tips were removed in two fragments, and sectioned transversely in two fragments, and explants were incubated onto the Pre-culture medium containing MS with 30 g/L Sucrose, 8 g/L plant agar, 1 mg/L 1-Naphthaleneacetic acid (NAA), 1 mg/L benzylaminopurine (BAP) with the adaxial sides down. Explants were co-cultured in the *Agrobacterium* suspension for 20 min and transferred to a sterile filter paper to briefly drain remaining *Agrobacterium* suspension, placed onto the same medium used for Pre-culture. Two days after, explants were transferred to Shoot induction medium containing MS with 30 g/L Sucrose, 8 g/L plant agar, 2 mg/L trans zeatin-riboside,

0.1 mg/L Indole-3-acetic acid (IAA), 10 mg/L hygromycin, and 250 mg/L carbenicillin. About 4 to 6 weeks after, explants were moved to Shoot elongation medium containing MS with 30 g/L Sucrose, 8 g/L plant agar, 1 mg/L trans zeatin-riboside, 0.1 mg/L IAA, 10 mg/L hygromycin, and 250 mg/L carbenicillin. About 1-2 cm shoots were cut and transferred to Rooting medium containing MS with 30 g/L sucrose, 8 g/L plant agar, 1 mg/L IAA, 10 mg/L hygromycin, and 250 mg/L carbenicillin.

Nucleic acid extraction and PCR screening

Genomic DNA (gDNA) was extracted from leaf samples of hardened regenerated plants by cetyltrimethylammonium bromid (CTAB) method (Porebski et al., 1997) with 3mm beads in a 1.5ml tube. Extracted DNAs were quantified by Nanodrop spectrophotometer (Nanodrop Technology, Inc., Wilmington, DE, USA) and diluted to a final concentration of 100 ng/µL.

Table 2. Components of plant tissue culture media

Media	Compositions	Days
Germination media	1/2 MS, 20g/L sucrose, 8g/L plant agar	6-7
Pre-culture media	MS, 30g/L sucrose, 8g/L plant agar, 1mg/L NAA, 1mg/L BAP	2
Co-culture media	MS, 30g/L sucrose, 8g/L plant agar, 1mg/L NAA, 1mg/L BAP	2
Shoot induction media	MS, 30g/L sucrose, 8g/L plant agar, 2mg/L trans zeatin riboside, 1mg/L IAA, 10mg/L hygromycin, 250mg/L carbenicillin	14-21
Shoot elongation media	MS, 30g/L sucrose, 8g/L plant agar, 1mg/L trans zeatin riboside, 1mg/L IAA, 10mg/L hygromycin, 250mg/L carbenicillin	60-80
Rooting media	MS, 30g/L sucrose, 8g/L plant agar, 1mg/L IAA, 10mg/L hygromycin, 250mg/L carbenicillin	14-21

Table 3. List of primers used in transgene confirmation

Name	Primer sequence (5’ to 3’)	Amplicon (bp)
HygR_F	GCGAAGAATCTCGTGCTTTC	209
HygR_R	CAACGTGACACCCTGTGAAC	209
Cas9_pHSE_F	ATCCAATCTTCGGCAACAT	484
Cas9_pHSE_R	TTATCCAGGTCATCGTCGTAT	484

Mutation detection

The transgenic plants were genotyped for mutation detection using primers flanking target regions. PCR products were purified using LaboPass PCR clean-up kit (Cosmo Genetech, Korea). The purified amplicons were also cloned into the TA cloning vector pMD20-T vector (Mighty TA-cloning kit, TAKARA) according to the manufacturer's instructions and selected by blue/white selection. White single colonies were inoculated in LB medium containing ampicillin. Plasmid was extracted using Accuprep plasmid mini extraction kit (Bioneer, Daejeon, Korea) and at least five clones per PCR product were sequenced using M13F and M13R primers (Bionics, Korea). Sequence alignments were performed using Lasergene's SeqMan program (DNASTAR, Madison, WI). The detection of mutations in E1 progeny was performed as described above.

Table 4. List of primers for mutation detection in target regions

Name	Primer sequence (5’ to 3’)	Amplicon size (bp)
Sl4E_F_2	ACACTATGGTCCAAACAGTTCTTAT	330
Sl4E_R_2	AACTGCTTGGGGAAGCTCAC	330
SlPDS275_F	TGCTTCTCAACATAAATCTTGACAAAGAGAAGGA	275
SlPDS275_R	CAAACCAAACCTTTAAAGGCCCCAAGT	275

Virus inoculations

For virus inoculum preparation, frozen stocks of TEV which was stored at -80℃. Ten days prior to disease screening, frozen inocula were inoculated to *Nicotiana benthamiana*. Frozen inocula were ground in 0.1M potassium phosphate buffer, pH 7.0, mixed with 400-grit carborundum, and rubbed on the 3rd leaves of *N. benthamiana*. After 10-20 minutes of inoculation, leaves were washed with distilled water (Hull., 2009). To inoculate the tomato plants, two pairs of cotyledons leaves were inoculated. Inoculated tomato plants were grown in a walk-in-chamber (16 hours light and 8 night under fluorescent light), and virus symptoms were observed by naked eye on non-inoculated upper leaves at 7 days of post-inoculation (DPI).

DAS-ELISA

At 7 Dpi, double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed for detecting coat protein (CP) of TEV three replicates of non-inoculated and infected leaves of E2 lines of 3-8 (E1), 3-11 (E1) lines were selected for the use of positive and negative controls in ELISA test. According to manufacturer's protocol (Agida, Elkhart, IN, USA), absorbance value of 405nm was measured in microplate reader (Anthon zenith 340 micro plate reader, UK)

RESULTS

gRNA design and the CRISPR/Cas9 system construction

To apply the CRISPR/Cas9 system and develop gene edited plants in tomato, I chose to target *phytoene desaturase* (*PDS* GenBank : EF650011) and *eukaryotic translation initiation factor 4E 1(eIF4E1* GenBank : AY723733*). PDS*, the key enzyme in carotenoid biosynthesis pathway, was chosen for testing genome editing efficiency due to their easily detectable phenotype change. *eIF4E* is known as a plant cellular translation factor essential for the Potyviridae infection. Also, mutation in the gene can confer resistance to potyviruses. In tomato, two genes (*eIF4E1* and *eIF4E2*) encode eIF4E proteins. To design gRNAs, the target gRNA sequences were identified using CCTop – CRISPR/Cas9 target online predictor (https://crispr.cos.uni-heidelberg.de/index.html). I chose gRNAs targeting in the first exon in the coding region of genes and scored high efficacy by prediction tool (Fig. 1). I decided to target the 5' region of the open reading frame (ORF), as mutations here would increase the likelihood of creating non-functional proteins by causing a coding frameshift or early stop codons. I designed two sgRNAs targeting +50 to +68 relative to the translation start site of *eIF4E1* gene and +26 to +44 relative to translation start site of *PDS* gene, respectively. These sgRNAs were cloned into a binary vector (pHSE401) which has a maize codon optimized Cas9 driven by 35S

promoter and the sgRNA driven by AtU6-26 promoters, respectively, together with the hygromycin resistance gene.

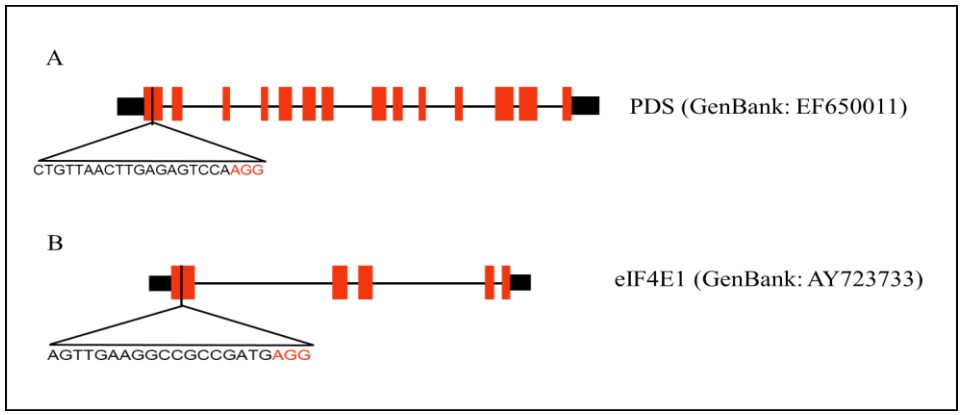

Figure 2. Schematic diagram of CRISPR/Cas9 target sites and gRNA sequences. (A) Schematic diagram of the CRISPR/Cas9 target site in the structure of *PDS*. (B) Schematic diagram of the CRISPR/Cas9 target site in the structure of *eIF4E1*. Each gRNA of target region is shown in black letter in followed red PAM.

Genome editing of *PDS*

To test the CRISPR/Cas9 system, pHSE401-PDS construct containing gRNA of PDS was delivered to *S. lycopersicum* Micro-Tom via *Agrobacterium*. The photo bleaching phenotype was observed in four out of 113 explants targeted for *PDS* (Fig. 3A) demonstrating that about the PDS gene of 4% explants were edited both alleles of the *PDS* gene.

White color shoots of *PDS* edited explants were sampled and the existence of the transgene was confirmed by PCR using *Cas9* and *Hpt* specific primers (Fig. 3B). Expected sizes of PCR products were obtained from gRNA of white color shoots demonstrating that the explants contained both *Cas9* and *Hpt* genes (Fig. 3B).

To confirm mutations in the *PDS* gene, PCR product amplified from the targeted region of *PDS* was sequenced by Sanger sequencing method. Sequence analysis showed two types of sequence variations, biallelic mutation: one-nucleotide deletion and 2-nucleotides deletion at the upstream of the PAM sequence (Fig. 4). These biallelic mutations resulted in the frame shift mutations causing early stop codons. As a result, the failure of expression functional PDS protein caused the photo bleaching phenotype demonstrating the CRISPR/Cas9 system can generate successful genome editing in target region in tomato.

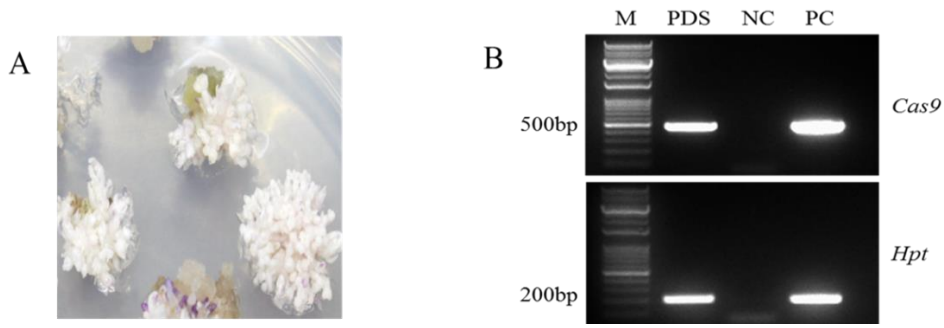

Figure 3. Phenotype and PCR analysis of *PDS* targeted explants. (A) *PDS* targeted explants representing photo bleaching phenotype. (B) PCR of *PDS* targeted plants expressing the *Cas9* gene and *Hpt* gene using gene specific primers from gDNA. NC: wildtype represented negative control; PC: *Cas9* plasmid for positive control.

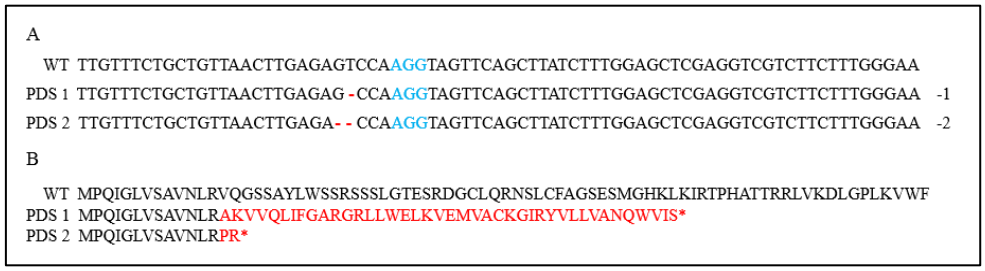

Figure 4. Mutation detection in *PDS* targeted explants by direct sequencing of PCR product in target region. (A) DNA sequence alignment between wild type and *PDS* transgenic. DNA deletions are shown by red dashes and deletion sizes (nucleotides) are marked on the right side of the sequence. Blue letter indicates PAM site. (B) Predicted amino acid sequence alignment *PDS* genes from genome-edited plants. The frame shift mutation caused early stop codons for disrupted protein are indicated with star symbol.

Development of *eIF4E1* edited tomato

To develop *eIF4E* edited tomato, pHSE401-eIF4E construct containing gRNA of *eIF4E1* was delivered to *S. lycopersicum* Micro-Tom via *Agrobacterium. eIF4E1* targeted explants were regenerated from callus, emerged shoots from callus were transferred to shoot elongation media. Elongated shoots were cut and transferred to rooting media for root formation. Rooted plants were transferred to soil for hardening (Fig. 5).

A total of 22 regenerated plants were obtained and the presence of transgenes, *Cas9* and *Hpt* genes was confirmed by PCR using transgene specific primers (Fig. 6). Seventeen out of 22 plants were identified to contain *Cas9* and *Hpt* genes demonstrating the transformation efficiency of 77% (Table 5).

Figure 5. *Agrobacterium* mediated tomato (cv. Micro-Tom) transformation.

eIF4E1 targeted tomato transformation developmental stages. From the left, germination of seeds, co-culture of explants, shoot induction from explants, shoot elongation, rooting plants, and transferred plants to soil.

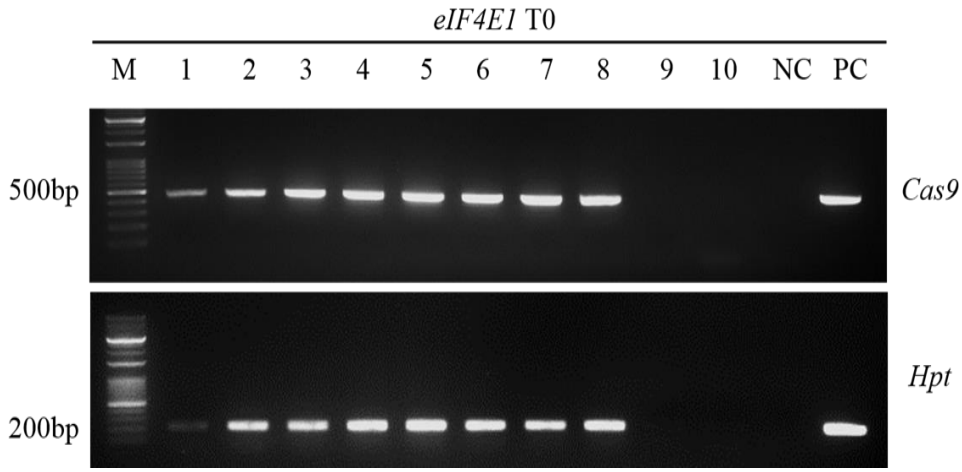

Figure 6. PCR screening of *S. lycopersicum* Micro-Tom transgenic lines.

PCR screening of *eIF4E1* targeted T_0 plants expressing the *Cas9* gene and *Hpt* gene from gDNA. NC: wildtype represented negative control; PC: *Cas9* plasmid for positive control.

Table 5. Summary of *eIF4E1* targeted genome editing

No. of regenerated plants	No. of transgenic plants	No. of transgenic plants containing *eIF4E* mutations
22	17 (77%)	16 (94%)

Mutation detection

To identify CRISPR/Cas9-induced mutations, PCR products amplified from gDNA of 17 transgene positive samples using gRNA flanking primers and the amplicons were sequenced. Sixteen out of 17 putative genome edited transgenic plants showed mixed sequence peaks at the target region of gRNA compared to the wild type target sequence (Fig. 7B). These results indicated that the target region might contain mutations induced by Cas9 and gRNA showing 94.1% efficiency of *eIF4E1* targeted genome editing (Table 5).

Among the *eIF4E1* mutants, E0-3 and E0-8 lines were randomly selected to generate E_1 plants. To characterize the mutation sequences, E0-3, and E0-8 transgenic plants were further investigated by sequencing the target region. PCR products were cloned and each clone was sequenced. Sequencing results showed that PCR product from E0-3 contained four different alleles of eIF4e the target region, whereas E0-8 contained five different alleles (Fig. 7).

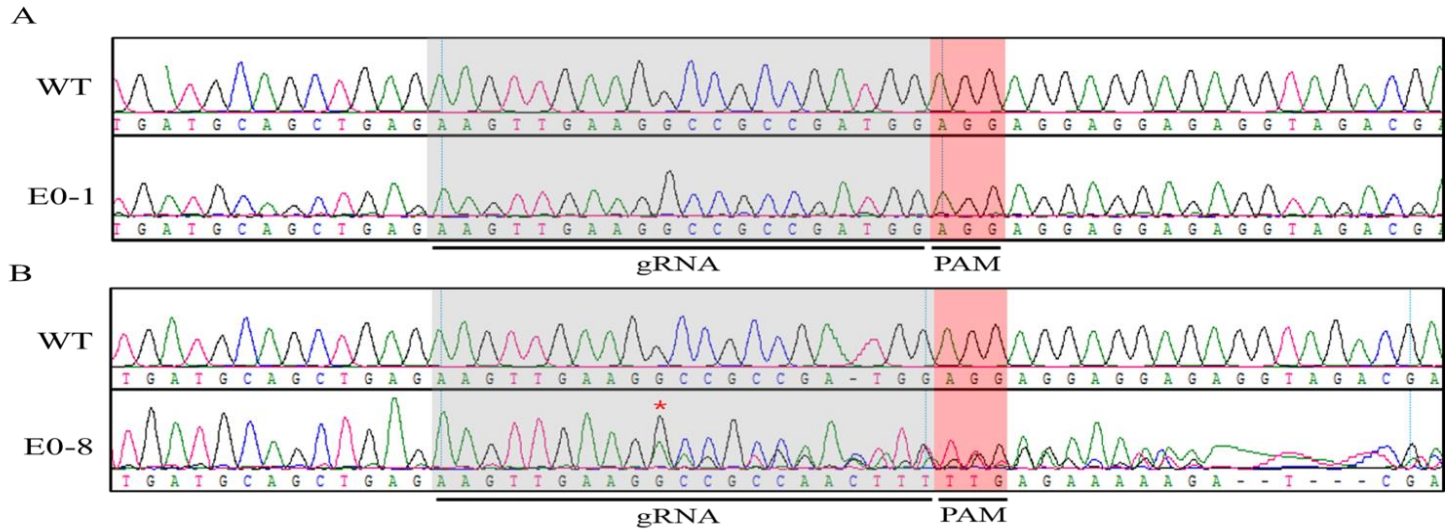

Figure 7. Mutation screening by PCR product direct sequencing. (A) Sequence alignment of *eIF4E1* targeted E0-1 line transgene inserted. It shows normal single peak of chromatogram in target region compared to wild type sequence. It regarded as a non-mutated plant. (B) Sequence alignment of *eIF4E1* targeted E0-8 line transgene inserted. It showed mixed peak in target region due to InDel compared to wild type sequence. It regarded as a mutant by CRISPR/Cas9 genome editing.

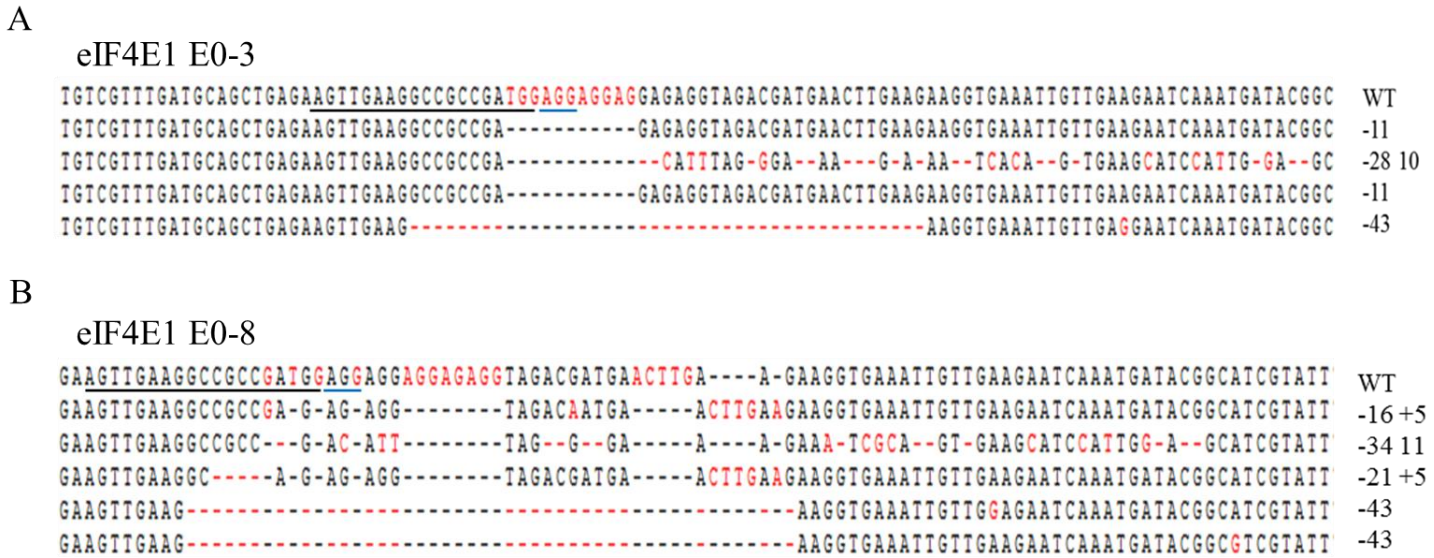

Figure 8. Mutation sequence alignment in E0. (A) Alignment of 4 colony sequences from *eIF4E1* E0-3 line with the wild type sequence. (B) Alignment of 5 colony sequences from *eIF4E1* E0-8 line with the wild type sequence.

Genotyping of E1 progeny

To obtain E_1 lines, E0-3 and E0-8 lines were self-pollinated and gDNA was extracted from leaf samples of E1 progeny. Presence of transgene were tested by PCR with the transgene specific primers. Among 19 plants derived from E0-3, 13 plants carried transgenes (#1, 2, 3, 4, 9, 10, 12, 13, 15, 16, 17, 18, and 19). Among seven plants from E0-8, five plants had transgenes (#3, 4, 5, 6, and 7) (Fig. 9). To reveal sequence variations of E_1 plants, amplicons of the target region in E_1 plants derived from E0-3 and E0-8 plants were sequenced. Representative sequences of homozygous, bialleleic, mixed alleles are shown (Fig. 10). E3-8 line contained a 20 bp deletion, E3-11 line had 43 and 11 bp deletions, and E3-19 line had 12, 13, and 15 bp deletions, and combined mutation.

To select homozygous mutants from E0-8 line, TA cloning of amplicon was conducted and more than 5 colonies per plant were sequenced. As a result, three E_1 lines (8-7, 3-8, and 3-17) that had 43 bp deletion, two lines (3-9 and 3-15) that showed 29 bp deletion and 10 nucleotides substitution were obtained (Fig 11). Among these lines, E_2 progeny of 3-8 line was generated by self-pollination and used for virus resistance analysis (Fig 12).

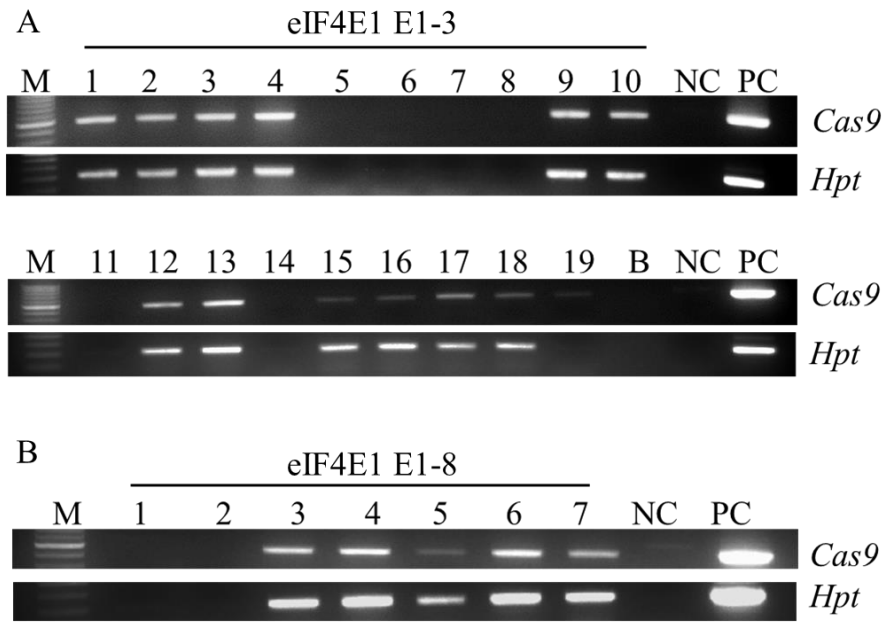

Figure 9. Genotyping of E_1 progeny.

(A) Transgene screening of *eIF4E1* targeted E_1 progeny of E0-3 by PCR (B) Transgene screening of *eIF4E1* targeted E_1 progeny of E0-8 by PCR

Figure 10. Representative genomic sequence of *eIF4E1* transgenic according to mutation pattern. (A) Homozygous mutation line (*eIF4E1* 3-8 (E_1)) (B) Biallelic mutation line (*eIF4E1* 3-11 (E_1)) (C) Chimeric mutation line (*eIF4E1* 3-19 (E_1)).

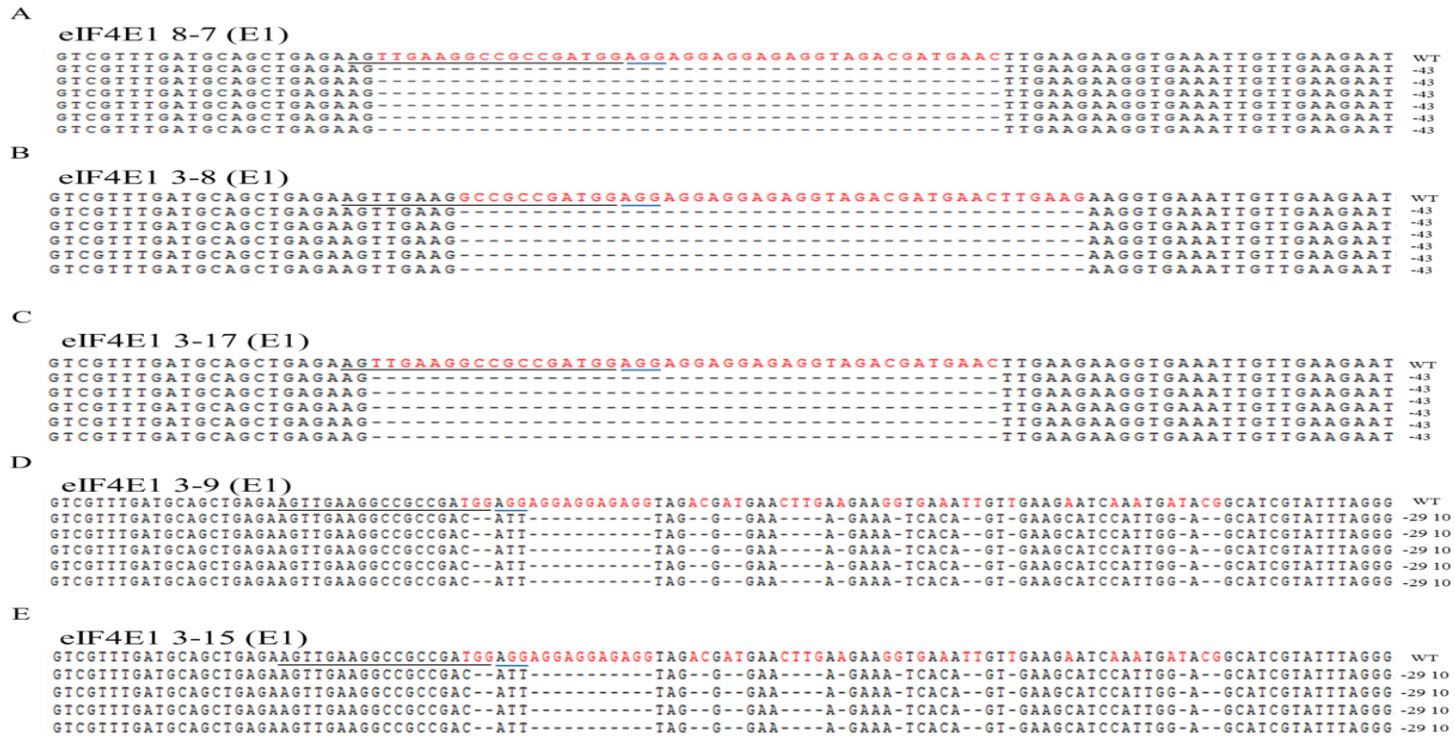

Figure 11. *eIF4E1* homozygous E_1 line sequence alignment. Total 5 homozygous lines were obtained. Three homozygous lines with 43 base pair deletion (Fig. 11A-C) and two homozygous lines with -29 base pair deletion and 10 base pair substitution (Fig. 11D-E) were identified through sequence analysis.

Figure 12. *eIF4E1* E_1 homozygous transgenic.

Virus resistance screening

To screen TEV-HAT resistance, E_2 progeny of 3-8 (homozygous line) and 3-11 (biallelic line) seedlings were used. Two cotyledons of 12-days-old tomato seedlings were inoculated with the TEV-HAT. At 7 days post inoculation, the wild type plant showed typical TEV symptoms including vein clearing and several small chlorotic spots in the leaves. Similar symptoms were observed in both *eIF4E* mutant lines (Fig 13). To confirm virus infection, DAS-ELISA analysis was done using systemic uninoculated leaves. The similar amount of virus coat protein was accumulated in E_2 progeny of E3-8 and E3-11 as the wild type plants indicating that *eIF4E* edited lines were susceptible to TEV-HAT (Fig 14).

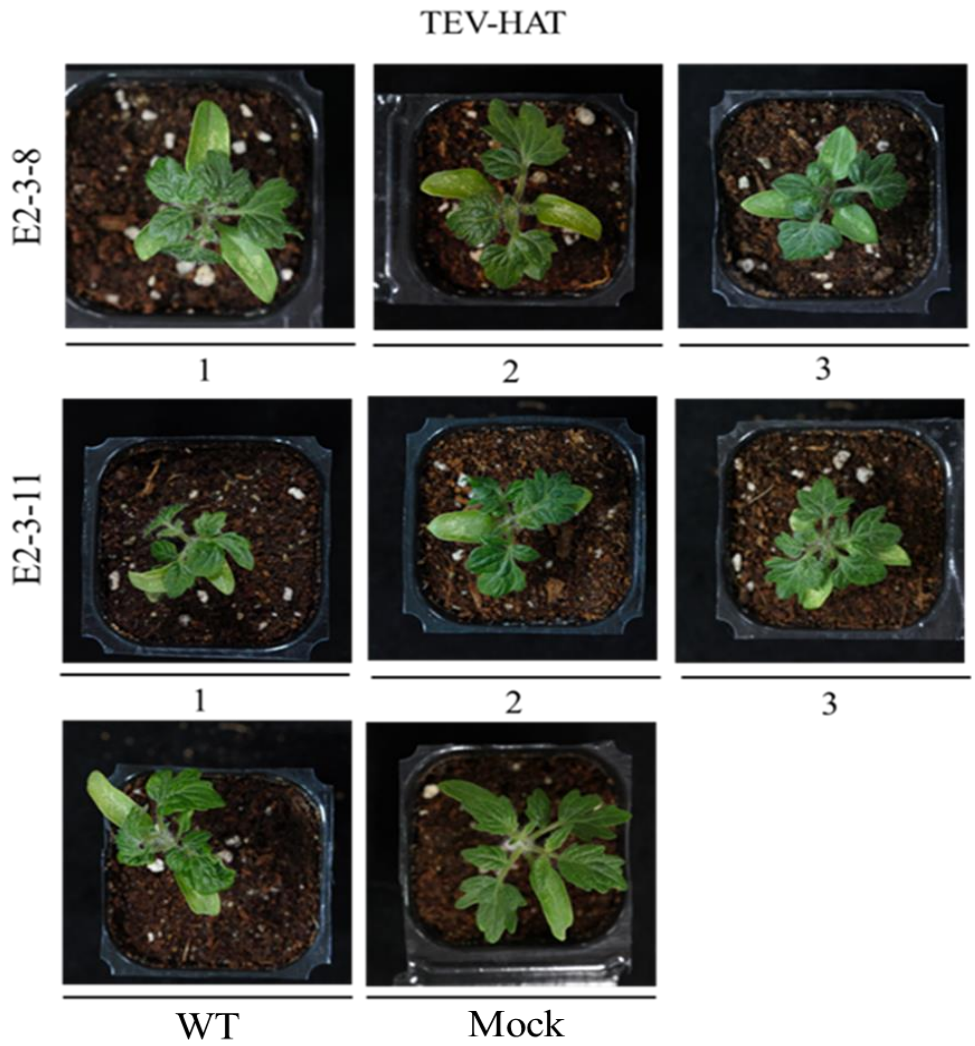

Figure 13. 7 DPI of TEV-HAT inoculated *eIF4E1* mutant lines.

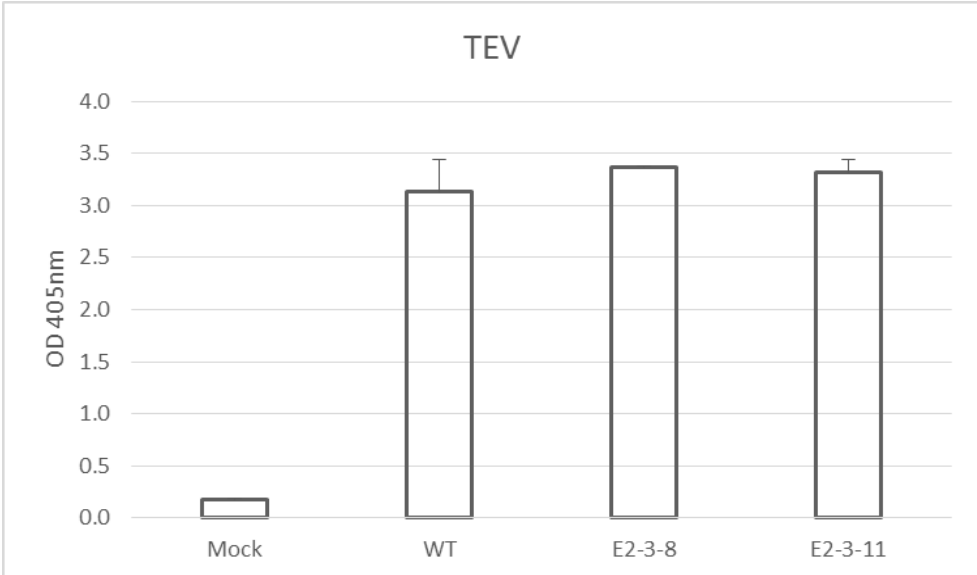

Figure 14. TEV-HAT resistance analysis by DAS-ELISA.

To analyze TEV-HAT resistance of *eIF4E1* mutants virus inoculated, DAS-ELISA was conducted.

DISSCUSSION

Plant biotechnology can develop plants faster than traditional breeding techniques. Many studies have been conducted regarding interaction between plants and viruses, which revealed essential host factors for virus infection. Mutations in these host factors render virus resistance in nature, which provides opportunities to develop novel virus resistance using genome editing methods. Recently, the emergence of a new gene editing technology, CRISPR/Cas9, has quickly begun to replace existing gene editing techniques such as ZFN and TALEN, with the advantage of being easy to manipulate and cost effective.

In this study, I conducted an experiment to edit the *eIF4E1* gene using CRISPR/Cas9 technology with an aim of creating potyvirus resistance in *S. lycopersicum* Mico-Tom. To validate the genome editing method in tomato, I first used the *PDS* gene as a target gene. The albino phenotype of transgenic tomato explant carrying *PDS* gRNA confirmed the function of *PDS* as previously reported (Zhang et al., 2016, Pan et al., 2016). The sequencing result of *PDS* demonstrated that DSB occurred at the 3 bp upstream of the PAM Site (NGG) resulting in deletions of the *PDS* gene, as reported previously (Garneau et al., 2010, Jinek et al., 2012). The deletion mutations led to early stop codons leading to gene knock-out.

The E_0 plants targeted for *eIF4E* were shown to have a mixture of different allelic variations, which is different from previous reports in Arabidopsis and rice

studies and similar to those reported in tomato (Ma et al., 2013; Zhang et al., 2014; Pan et al., 2016). In the previous genome editing studies, diverse mutation patterns including insertion, deletion, substitution, and combined mutation with no wild type allele were observed in the E_0 generation. This can be inferred that the Cas9 activity is continuously active in the presence of the wild type allele creating new alleles (Pan et al., 2016).

E_0 plants carrying diverse alleles in the target gene were advanced to the next generation, and E_1 lines carrying homozygous alleles could be selected from segregating populations. In addition, E_1 null segregants without having *Cas9* transgene were selected from the segregating population (Xu et al., 2015, Pyott et al., 2016, Chandrasekaran et al., 2016).

Using E_2 progeny derived from the homozygous E_1 lines without *Cas9* transgene, virus resistance to potyviruses was tested. E_1 plants carrying mutations in *eIF4E* showed typical TEV symptoms. DAS-ELISA analysis also showed TEV accumulation resistance in the tested GE plants, homozygous E2-3-8 and bialleic lines E2-3-11 lines. This may be due to redundant activities of *eIF4E* homologs present in tomato. Similar results were obtained when an *eIF4E1* knockout tomato plant selected from a TILLING population was tested for potyvirus resistance demonstrating a narrower range of resistance rather than the broad spectrum of viral resistance seen in the wild accession *S. habrochaites* PI24. The *eIF4E1* knockout plants showed a tendency to increase the *eIF4E2* gene expression, and did not show resistance to TEV, which uses both *eIF4E1* and *eIF4E2* genes. By contrast, *eIF4E1*

and *eIF4E2* double knockout plants showed a broad spectrum resistance to a wide range of potyviruses, while growth and development of the double knockout plants were retarded (Gauffier et al., 2016). Therefore, development of a novel virus resistance tomato line can be a challenge due to the redundancy of *eIF4E* homologs.

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ABSTRACT IN KOREAN

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) 기술의 개발로 거의 모든 유기체에서 유전체 편집을 가능하게 되었다. Eukaryotic translation initiation factor 4E (eIF4E)는 식물의 열성 바이러스 저항성 유전자에 의해 암호화된 단백질이다. 본 연구에서는 토마토 (*Solanum lycopersicum* cv. Miro-Tom) 에서 유전자교정 (GE)의 대상으로 *eIF4E* 유전자를 선정하였다. *phytoene desaturase* (PDS) 또는 *eIF4E* 유전자를 목표로 하는 gRNA와 *Cas9* 유전자가 아그로박테리움 매개 방법으로 Miro-Tom에 형질전환하였으며, *Cas9*과 gRNA가 삽입된 토마토 형질전환체를 얻었다. 전체 113개 중 4개에서 알비노 표현형을 나타내는 유전자교정 식물체를 T_0세대에서 얻을 수 있었다. *eIF4E* 유전자교정 토마토를 얻기 위해, *eIF4E* 형질전환체의 염기서열 분석을 실시하였다. 총 16개의 형질전환체 중 11개에서 43 베이스페어의 범위의 다양한 결실을 가지는 E_0 식물체를 얻었다. E_0 식물체들은 자가수분을 하였으며, 5개 *eIF4E1* 돌연변이체를 얻었다. E_2 세대에 *Tobacco etch virus* (TEV) 접종을 실시하였다. *eIF4E1* 돌연변이체에서 TEV에 저항성을 보이지 않았으며, 이는 토마토 *eIF4E* 유전자에 존재하는 유전자중복에 따른

결과로 예상할 수 있었다. 본 연구는 CRISPR/Cas9 기술은 목표하고자 하는 유전자에 돌연변이를 일으키는 유용한 도구로 사용될 수 있음을 보여준다.

주요어: 토마토, 유전자 편집, 포티바이러스, CRISPR/Cas9, Eukaryotic translation initiation factor 4E (*eIF4E*), Phytoene desaturase (*PDS*)

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