

SHORT COMMUNICATION

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A Variant of LbCas12a and Elevated Incubation Temperatures Enhance The Rate of Gene Editing in the Oomycete *Phytophthora infestans*

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CRISPR-Cas editing systems have proved to be powerful tools for functional genomics research, but their effectiveness in many non-model species remains limited. In the potato and tomato pathogen *Phytophthora infestans*, an editing system was previously developed that expresses the *Lachnospiraceae* bacterium Cas12a endonuclease (LbCas12a) and guide RNA from a DNA vector. However, the method works at low efficiency. Based on a hypothesis that editing is constrained by a mismatch between the optimal temperatures for *P. infestans* growth and endonuclease catalysis, we tested two strategies that increased the frequency of editing of two target genes by about 10-fold. First, we found that editing was boosted by a mutation in LbCas12a (D156R) that had been reported to expand its catalytic activity over a broader temperature range. Second, we observed that editing was enhanced by transiently incubating transformed tissue at a higher temperature. These modifications should make CRISPR-Cas12a more useful for interrogating gene and protein function in *P. infestans* and its relatives, especially species that grow optimally at lower temperatures.

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Keywords: CRISPR-Cas editing, *Lachnospiraceae* bacterium Cas12a, *Phytophthora infestans*

CRISPR-Cas systems for editing genomes are formidable tools for research due to their ability to modify specific sites in DNA and adaptability to diverse species (Anzalone et al., 2020; Cai et al. 2022; Wang and Doudna 2023). Borrowed from components of bacterial adaptive immune systems, most CRISPR-Cas methods use an RNA-guided Cas endonuclease to generate a double-strand break (DSB) downstream of a short sequence named the protospacer adjacent motif (PAM). The DSB may be repaired by the cell's nonhomologous end-joining (NHEJ) path-

way or by homology-directed repair (HDR) in the presence of a template. NHEJ repair is imprecise, often introducing frameshift mutations that knock out gene function, while HDR has more diverse applications that also include replacing genes and tagging proteins. The first CRISPR editor to be deployed widely was Cas9, followed by Cas12a (Cpf1) (Zetsche et al. 2015). Natural and human-engineered variants have been identified that recognize new PAM sites, exhibit greater sequence specificity, or repress transcription without nicking the target (Anzalone et al. 2020; Cai et al. 2022; Wang and Doudna 2023).

In studies of plant-microbe interactions, CRISPR-Cas methods have helped to obtain insight into mechanisms of virulence, symbiosis, and host defense and to construct plants resistant to pathogens (Brauer et al. 2020; Butler et al. 2021; Li et al. 2022; Zhang et al. 2020). However, the efficiency of editing can be poor in some species, hampering research progress. One challenge is that the optimal temperature for Cas endonuclease activity may be unsuited to the organism. For example, since the oft-used SpCas9 originates from *Streptococcus pyogenes*, which grows best at 40°C, that enzyme is usually less effective in species that must be propagated at lower temperatures. Temperature-dependence is also exhibited by the Cas12a family, which comes from bacteria that grow best between 35 and 40°C (Haas and Blanchard 2017). Indeed, studies in cotton, rice, and zebrafish reported that editing by Cas12a from *Lachnospiraceae* bacterium (LbCas12a) was more frequent in the 28 to 34°C range than at lower temperatures such as 22°C (Banakar et al. 2021; Li et al. 2021; Malzahn et al. 2019). LbCas12a nevertheless maintains more of its activity at lower temperatures than does SpCas9 (Malzahn et al. 2019; Moreno-Mateos et al. 2017). This may make LbCas12a better suited for use in ectothermic animals, many plants, and some microbes.

The goal of the current study was to improve gene editing in the oomycete *Phytophthora infestans*, a pathogen that causes billions of dollars of losses on potato and tomato each year (Haverkort et al. 2008). Our aim was to address the discordance between the optimal temperatures for *P. infestans* growth and for the endonuclease activity of Cas proteins. While most strains of *P. infestans* grow best around 21°C (Johnson et al. 2015; Judelson et al. 2010), DNA cleavage by Cas9 and Cas12a occurs maximally at or above 37°C, based on in vitro assays (Kleinstiver et al. 2020; Xiang et al. 2017). Nevertheless, we were able to develop a transformation-based editing system for *P. infestans* using LbCas12a (Ah-Fong et al. 2021). Our experiments against several genes have resulted in frequencies of between 0 and 10%. Other researchers have also reported a low efficiency of editing by Cas12a in *P. infestans* (Wang et al. 2023). This contrasts with much higher frequencies, typically

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Both pSTU-1 and pSTUC-1 are available upon request.

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between 30 to 100%, reported for SpCas9 in other members of the genus such as *P. capsici*, *P. palmivora*, and *P. sojae* (Fang et al. 2017; Gumtow et al. 2018; Miao et al. 2018; Peng et al. 2019; Pettonghkao et al. 2020; Wang et al. 2022). Notably, each of these grow optimally at or above 28°C, which is well above the temperature preferred by *P. infestans* (Erwin and Ribeiro 1996). Higher rates of editing have also been obtained for both SpCas9 and LbCas12a in many plants, fungi, and animals, which are also more warm-adapted than *P. infestans* (Cui et al. 2022; Malzahn et al. 2019; Ouedraogo and Tsang 2020). SpCas9 has not been useful, so far, in *P. infestans*, apparently due to its toxicity (Ah-Fong et al. 2021).

In this study, we test two hypotheses related to editing in *P. infestans*. One is that editing would increase if higher incubation temperatures were used after LbCas12a-expressing plasmids were introduced into cells. The second hypothesis is that editing would be enhanced using a more temperature-tolerant variant of Cas12a. A prior study in *Arabidopsis thaliana* found that a D156R mutation in LbCas12a expanded its temperature range and increased editing two- to sevenfold at 22°C (Schindele and Puchta 2020). This also enhanced editing in *Drosophila melanogaster* at 25°C (Port et al. 2020). Based on such findings, we introduced the D156R mutation into LbCas12a in our editing plasmid, pSTU-1. This expresses an oomycete-adapted LbCas12a and guide RNAs (gRNAs), in a single transcriptional unit, plus a separate drug-resistance gene for selecting transformants (Fig. 1A). The modification to LbCas12a was made using the QuikChange II site-directed mutagenesis kit (Agilent, Santa Clara, CA, U.S.A.) with the primers in Supplementary Table S1; additional details about this and other procedures in this paper are presented in the Supplementary Material. The modified plasmid, pSTUC-1 (GenBank accession OR142980), was then used to express gRNAs against genes PITG_10768 and PITG_03290, which encode zinc finger and E2F transcription factors, respectively. Our preliminary studies indicated that these genes could be edited or transcriptionally silenced without affecting growth, so both should represent relatively neutral targets for testing modifications to the Cas12a system. gRNAs targeting the open reading frames of the genes were designed using EuPaGDT (Peng and Tarleton 2015), with the length parameter set to 23 nt and the PAM to 5'-TTTV. Ar-

rays of two gRNAs for PITG_10768 and four for PITG_03290, respectively, were developed based on the principle that multiple gRNAs should boost the likelihood of editing (Supplementary Tables S1 and S2). These arrays were inserted into pSTU-1 and pSTUC-1 by ligating annealed oligonucleotides into their *BsaI* sites (Figs. 1B and 2A). DNA for transforming the resulting four plasmids into *P. infestans* was then prepared using the Macherey-Nagel purification kit (Takara Bio, San Jose, CA, U.S.A.).

Our first experiment tested whether LbCas12a^{D156R} resulted in higher rates of editing than normal (D156) LbCas12a, using gene PITG_10768 as a target. The vectors were introduced into diploid strain 1306 by protoplast transformation (Ah-Fong et al. 2008). After DNA treatment, the protoplasts were incubated in nonselective broth at 25°C for 24 h, to allow for cell-wall regeneration and the initiation of hyphal growth. The tissue was then plated on agar media containing 8 µg of G418 per microliter at room temperature (21 to 22°C), with colonies appearing after 6 to 7 days. This deviates from our published protocols for transformation, which call for incubating the material at 18°C (Ah-Fong et al. 2008). Although hyphal growth is reduced by 39% at 25°C (Judelson et al. 2010), we anticipated that the warmer conditions might enhance editing; the effect of temperature will be addressed directly later in this paper. After transferring transformants to new selection plates, mutations were detected using genomic DNA isolated from 4 day-old cultures by phenol-chloroform extraction (Ah-Fong et al. 2021). The targeted regions were then amplified by polymerase chain reaction (PCR) using flanking primers (Supplementary Table S1). Sanger sequencing was performed using DNA cleaned with the EXOSAP-IT Express reagent (ThermoFisher, Waltham, Massachusetts, U.S.A.). Cases where no PCR product was obtained were resolved by either re-extracting genomic DNA, using primers farther from the targeted chromosomal site, or both. Editing was indicated by a shift in amplicon size or a sequence change. Double peaks in Sanger chromatograms would indicate that one allele was edited, while a homogenous sequence different from wild type would indicate biallelic editing or monoallelic editing followed by gene conversion.

This first experiment demonstrated that LbCas12a^{D156R} resulted in a fourfold increase in editing compared with normal LbCas12a, using PITG_10768 as the target. Each comparison

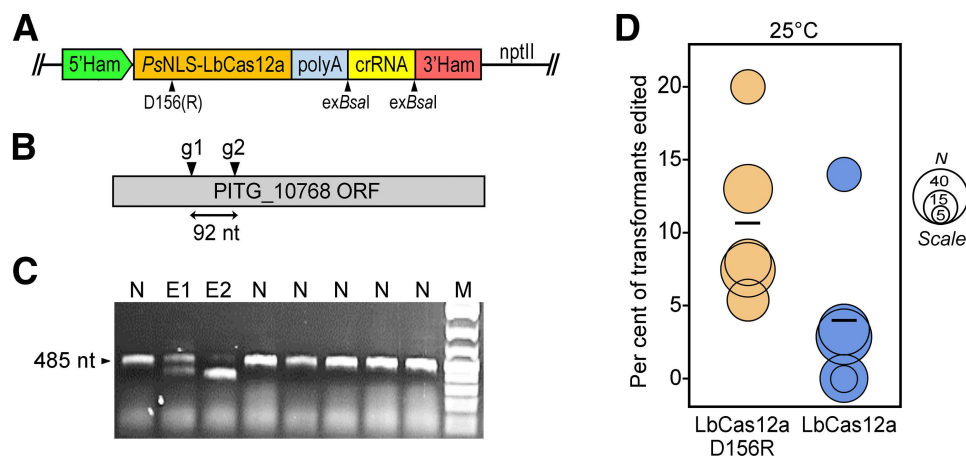


Fig. 1. Influence of the D156R mutation on editing of gene PITG_10768. **A**, An abbreviated schematic of plasmids pSTU-1 and pSTUC-1, the latter containing the D156R mutation. **B**, Location of guide RNAs within the target gene. **C**, PCR analysis of targeted regions in transformants. Nonedited and edited strains are marked with an N and E, respectively, and M is the molecular weight marker (GeneRuler 1K Plus ladder, ThermoFisher). The allele yielding the faint 485-nt band in lane E2 was not detected in Sanger sequencing, which suggests either that this band was due to spillover from adjacent lanes during gel loading or that the culture initially contained a low proportion of unedited nuclei. This emphasizes the importance of performing single-zoospore (single-nucleus) purification of edited strains that will be used for functional genomics. **D**, Bubble plot showing editing efficiencies achieved with each enzyme, using the 25°C regeneration temperature. Each circle represents an individual transformation, with its volume corresponding to the number of transformants analyzed. The mean frequency of editing is shown as a black horizontal bar.

in this and subsequent studies was done in parallel and was performed four or five times spread across several weeks. The results from each of these biological replicates are shown by separate bubbles in Figure 1D, with the size of each bubble indicating the number of transformants analyzed; the underlying numerical data are provided in Supplementary Table S3. Of 117 transformants obtained with LbCas12a^{D156R}, 11.1% were edited, compared with only 2.6% of 116 transformants generated using normal LbCas12a (Fig. 1D; Table 1). This is significantly different based on Fisher's exact test ($P = 0.0172$).

Analyses of the transformants edited for PITG_10768 by DNA sequencing and gel analysis of PCR products indicated that 63% included deletions of approximately 90 nt. Since the targets of the two gRNAs were 92 nt apart (Fig. 1B, g1 and g2), this suggested that NHEJ had occurred after both sites had

been cleaved by LbCas12a. In 37% of the edited strains, smaller deletions (<25 nt) were observed, which was consistent with cleavage of the chromosome at a single site. Examples of two of the large deletion events are presented in Figure 1C. Lane E1 portrays an apparent monoallelic event, since the strain contains a 485-nt wild-type PCR band plus one reduced by about 92 nt, the distance between the two gRNAs. Lane E2 shows an example of biallelic editing, in which only a single band reduced by about 92 nt was revealed by PCR. Biallelic editing may result from the nicking and repair of both alleles by LbCas12a or by gene conversion following a single-allele event. Overall, one-quarter of the edited transformants had experienced biallelic editing, and its frequency was not significantly different between the two endonucleases. The fraction of transformants exhibiting editing varied substantially between days, ranging from 5.3 to 20% in the case of LbCas12a^{D156R}.

Our next experiment addressed both the utility of the D156R mutation and the influence of temperature on editing, targeting gene PITG_03290. For temperature comparisons, protoplasts were treated with DNA in a single tube and split immediately into three tubes. These were incubated in media at 18, 25, or 28°C for 24 h, and then on selective media at 21 to 22°C. We limited the initial incubation step to 24 h, since most editing events seem to occur soon after transformation, based on a high frequency of homokaryons among edited strains (Ah-Fong et al. 2021). Moreover, a short period at a higher temperature was enough to increase editing by at least fivefold in *Arabidopsis* and citrus (LeBlanc et al. 2018). We did not test temperatures higher than 28°C, since *P. infestans* growth ceases at 30°C; at 28°C, growth is reduced by 59%.

The results of this experiment with PITG_03290 confirmed that the use of the D156R variant enhanced editing ($P = 9.7 \times 10^{-6}$, one-way analysis of variance). For example, at 28°C the percentage of transformants mutated by LbCas12a^{D156R} was 25% versus only 2.1% for normal LbCas12a (Fig. 2B; Table 1). Moreover, the 24-h 25- and 28°C regimes both increased editing compared with the treatment in which the regenerating protoplasts were incubated at 18°C ($P < 0.05$, for both enzymes). For example, the frequency of editing using LbCas12a^{D156R} was at least twofold higher at 25 and 28°C compared with 18°C.

Deletions in PITG_03290 between two of the four gRNA targets were more common than small deletions, representing 71% of the edited strains. This is similar to the situation observed with PITG_10768. Examples of such deletions (approximately 105 and 295 nt) are displayed in Figure 2C. Biallelic events are shown in lanes E1, E3, and E5, while lanes E2 and E4 illustrate monoallelic events. As with PITG_10768, the frequency of editing varied up to 10-fold between experiments performed on different days (Fig. 2B; Supplementary Table S3). This variation appeared to be independent of the batch of DNA, age of the DNA, or regeneration frequency of the protoplasts.

In summary, one major conclusion from our experiments is that LbCas12a^{D156R}, combined with a higher post-transformation incubation temperature, causes more than a 10-fold increase in editing compared with our prior protocol. Much of this is likely due to increased catalytic activity of the endonu-

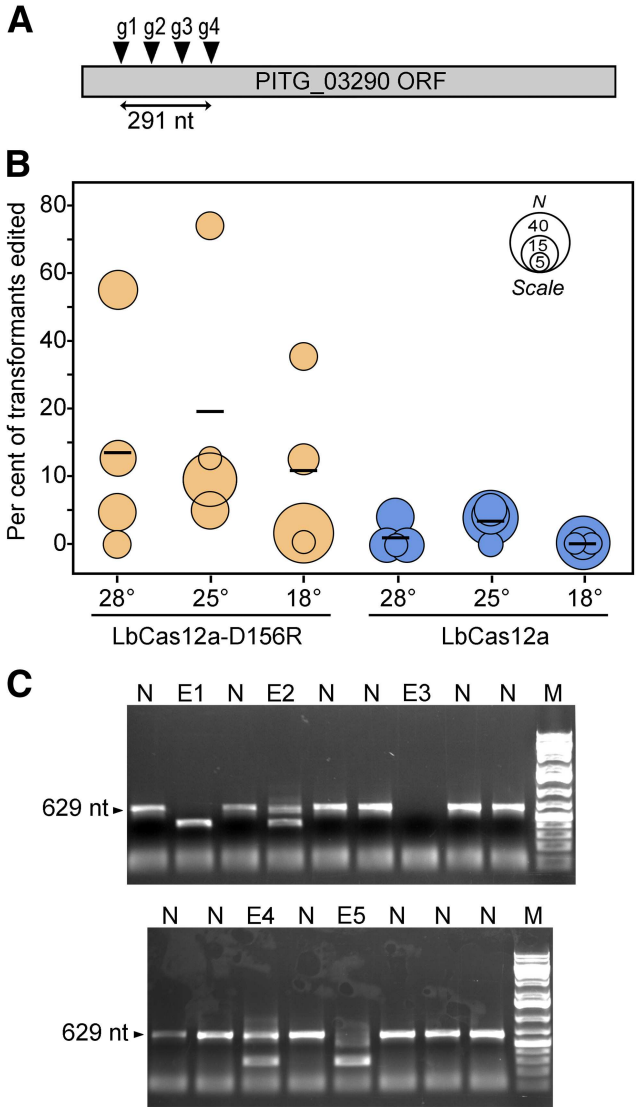


Fig. 2. Influence of the D156R mutation and regeneration temperature on editing of gene PITG_03290. **A**, Location of guide RNAs within the gene. **B**, Bubble plot showing editing efficiencies achieved with each enzyme, using the indicated regeneration temperatures. Each circle represents an individual transformation, with its volume corresponding to the number of transformants analyzed. The mean frequency of editing is shown as a black horizontal bar. **C**, PCR of targeted regions in transformants. Nonedited and edited strains are marked with N and E, respectively, and M represents the molecular weight marker. The strain marked E3 has a large deletion that eliminated one of the primer binding sites used for this amplification.

Table 1. Summary of editing frequencies from all experiments

Target gene	Temperature (°C)	Percent of transformants edited	
		LbCas12a	LbCas12a-D156R
PITG_10768	25	2.6	11.1
PITG_03290	18	0.0	10.9
	25	6.0	22.0
	28	2.1	25.0

clease, but temperature may also influence other factors that affect editing, such as chromatin structure or the NHEJ pathway.

A second important observation from this study is that arrays of multiple gRNAs often result in large deletions between their target sites in *P. infestans*, which could be useful for removing whole genes or chromosomal segments. Related strategies have succeeded in non-oomycetes (Cui et al. 2019; Kissling et al. 2019). This would also accelerate experimentation, since editing could be detected by gel electrophoresis alone, without sequencing. These benefits of using arrays are in addition to the value of hedging against the possibility that a single gRNA might be ineffective due to its secondary structure or inaccessibility of the chromosomal target (Thyme et al. 2016). Cas12a is especially suited to the array approach, since its crRNA is much smaller than that used by Cas9 (Zetsche et al. 2015).

Our major conclusions have been confirmed through studies of two genes, in experiments that compared variables using the same gene. The latter point is important, since distinct genes or sites within genes may vary in their susceptibility to editing due to variation in chromatin accessibility, the binding of competing proteins, or the gene's contribution to fitness (Strohkendl et al. 2021; Thyme et al. 2016; Verkuijl and Rots 2019; You et al. 2020). Thus, conclusions from comparing results against different genomic targets could be misleading. Indeed, our improvements that enhanced editing of PITG_03290 and PITG_10768 by five to 10-fold yielded frequencies only twofold higher than that reported with our original protocol against *inf1*, also known as PITG_12551, as the target (Ah-Fong et al. 2021). This might be explained by the relative transcriptional activity of the three genes. Based on RNA-seq, PITG_12551 is one of the most actively transcribed genes in *P. infestans*, having messenger RNA levels in hyphae that are 100-fold higher than PITG_03290 and PITG_10768, which exhibit near-median levels of expression (Ah-Fong et al. 2017). At least for Cas9, RNA polymerase has been suggested to speed the release of the endonuclease from the cut ends of the chromosome, facilitating repair (Clarke et al. 2018). Such issues, including variation in chromatin structure, may also explain why the frequency of editing was twofold higher for PITG_03290 compared with PITG_10768, although the fact that double the number of gRNAs were used against the former may also be a reason.

Our current study has also illustrated the importance of drawing conclusions from biological replicates, since the rate of editing for both genes in this study varied significantly between experiments. We suggest that this might be due to factors related to the dynamics of transformation. These might include the number of DNA molecules taken up per protoplast or the stage of the nuclear cycle at which protoplasting took place, since editing may be cell cycle-dependent (Miskel et al. 2022). Identifying such factors should lead to additional improvements in CRISPR-Cas technology for *Phytophthora* spp. While substantial experiment-to-experiment variation in the frequency of editing is not generally reported in plants (Lin et al. 2017), this does occur in mammalian systems that use delicate transformation methods such as microinjection (Midic et al. 2017).

Although our experiments have focused on *P. infestans*, the improvements described in this paper should be useful for other oomycetes. They may be especially helpful in the one-quarter of the genus *Phytophthora* that, like *P. infestans*, grow optimally below 25°C (Erwin and Ribeiro 1996). This includes many important species, such as the strawberry pathogen *P. fragariae*, the broad-host range pathogen *P. megasperma*, and the invasive tree pathogen *P. kernoviae*. Our approach may also be useful for psychrotrophic fungi, many nematodes, and extremophilic prokaryotes, combined with further engineering and understanding of the components of the editing system.

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