Attempts to implement CRISPR/Cas9 for genome editing in the oomycete Phytophthora infestans

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Abstract

Few techniques have revolutionized the molecular biology field as much as genome editing using CRISPR/Cas9. Recently, a CRISPR/Cas9 system has been developed for the oomycete Phytophthora sojae, and since then it has been employed in two other Phytophthora spp. Here, we report our progress on efforts to establish the system in the potato late blight pathogen Phytophthora infestans. Using the original constructs as developed for P. sojae, we did not obtain any transformants displaying a mutagenized target gene. We made several modifications to the CRISPR/Cas9 system to pinpoint the reason for failure and also explored the delivery of pre-assembled ribonucleoprotein complexes. With this report we summarize an extensive experimental effort pursuing the application of a CRISPR/Cas9 system for targeted mutagenesis in P. infestans and we conclude with suggestions for future directions.

Introduction

The oomycete Phytophthora infestans, the causal agent of potato and tomato late blight, is an economically important plant pathogen that is difficult to control (Kamoun et al. 2015). Current efforts to identify and functionally analyze genes important for virulence, are hampered by a limited molecular toolbox. Gene knock-outs (KOs) or gene deletions are not possible via homologous recombination, as transgenes are integrated randomly via non-homologous end-joining (NHEJ) (Judelson 1997; Fang & Tyler 2016). Moreover, P. infestans is diploid and heterothallic. Generating sexual progeny is quite challenging and as a result studies based on genetic analyses are scarce (Govers & Gijzen 2006; Fry 2008). Consequently, to date, functional gene studies in oomycetes primarily rely on gene silencing and overexpression. DNA transformation of the target gene in P. infestans can lead to homology dependent gene silencing or overexpression (van West et al. 1999). By fusing a fluorescent tag to the target gene, the overexpression transformants can also be exploited for investigating the subcellular localization of the encoded protein. However, due to the random integration of transgenes and varying levels of silencing and overexpression efficiency, phenotypes often vary between transformed lines, experiments, and labs (Fang & Tyler 2016).

Recently, a CRISPR/Cas9 genome editing system has been developed for Phytophthora sojae (Fang & Tyler 2016). This provides an important addition to the molecular toolbox for oomycetes, and makes it up to par with other research areas. For genome editing based on CRISPR/Cas systems, a nuclease, e.g. Cas9, is targeted to the desired DNA sequence by a so-called synthetic guide RNA (gRNA). The 20-nucleotide protospacer directs Cas9 to a specific target DNA site, which must be immediately 5′ of a protospacer adjacent motif (PAM) sequence (Ran et al. 2013). When Cas9
is directed to the target site a double-stranded break (DSB) is induced, which can be repaired either by the NHEJ repair mechanism, or via homology-directed repair (HDR) in case a repair template is available (Ran et al. 2013). Because of its low fidelity, NHEJ often leads to insertions or deletions (indels) in the repaired target gene, which can lead to frameshift mutations. HDR, on the other hand, can be exploited to introduce specific modifications such as base substitutions, insertions, or gene deletions.

So far, the P. sojae CRISPR/Cas9 system has been successfully used to mutagenize several genes in P. sojae (Fang & Tyler 2016; Ma et al. 2017), Phytophthora capsici (B.M. Tyler, personal communication), and Phytophthora palmivora (Gumtow et al. 2017). However, as of to date, no one has reported successful implementation of the system in P. infestans. Here, we present a case study for the effectuation of CRISPR/Cas9 for targeted genome editing in P. infestans. We chose to target three genes in P. infestans for a proof-of-principle study of CRISPR/Cas9-based genome editing in this organism.

The first target, Avr1, encodes an RXLR effector protein recognized by the cognate receptor protein R1 in potato. Recognition of AVR1 by R1 provides resistance to potato, while P. infestans strains lacking Avr1 are virulent on potato cultivars harboring the resistance gene R1. Moreover, R1 fails to recognize several mutated forms of AVR1 (Du et al. 2018). Hence, P. infestans Avr1 KO lines are expected to gain virulence on R1 potato, providing a clear phenotype compared to non-edited lines. The second target, PiTubA2, is one of the five α-tubulin encoding genes in P. infestans. Our aim was to modify the endogenous PiTubA2 gene in such a way that it encodes a fusion protein that has a GFP tag fused to the N-terminus of PiTUBA2. We therefore designed a HDR construct for gene knock-in at the endogenous PiTubA2 locus. A PiTubA2-GFP knock-in line would be ideal for live cell imaging of the microtubule cytoskeleton in P. infestans, as it lacks possible undesirable side effects associated with overexpression and/or ectopic integration of the transgene. The third target, PiAP5, encodes an aspartic protease (AP) with an C-terminal GPCR domain (Kay et al. 2011), a protein which is unique for oomycetes (van den Hoogen et al. 2018). To study to what extent the GPCR domain is important for functioning of the AP domain, we aimed at targeting Cas9 to the central region of the gene that separates the parts encoding the AP domain and the GPCR domain. An indel in this region should result in a truncated protein, which contains the N-terminal AP domain but lacks the C-terminal GPCR domain.

In the initial setup of our experiments, we used the CRISPR/Cas9 system as developed by Fang and Tyler (2016). In this system, the expression of Cas9 is driven by the strong constitutive Bremia lactucae Ham34 promoter and the expression of the gRNA by the P. sojae RPL41 promoter. The RPL41 promoter was chosen because the U6 small nuclear RNA promoters typically used in CRISPR/Cas9 systems, did not yield any detectable transcript (Fang et al. 2017). To ensure correct release of the gRNA from the transcript, the gRNA sequence was flanked with a hammerhead and a hepatitis delta virus (HDV) ribozyme at the 5’ and 3’ side, respectively (Fang et al. 2017).

This report summarizes an extensive experimental effort pursuing the application of a CRISPR/Cas9 system for targeted mutagenesis in P. infestans and concludes with suggestions for future directions.

Results and discussion

Experimental setup: design of gRNAs

The first, and perhaps most critical step of setting up a CRISPR/Cas9 experiment, is the design of the gRNAs. The genes of interest were screened for target sites using NGG as the PAM. All gRNAs were scored for on-target activity according to Doench et al. (Doench et al. 2014) and for off-targets interactions according to Hsu et al. (Hsu et al. 2013). The top 10 best scoring protospacers were manually curated using BLAST analysis on the P. infestans reference genome and predictions of the secondary RNA structure of the corresponding gRNAs. For Avr1, we selected the three best scoring gRNAs (Figure 1a), i.e. no predicted off-target interactions and no strong secondary RNA structure. For PiTubA2 and PiAP5 we selected the highest scoring gRNA (Figure 1b, 1c).

HDR constructs

To employ the HDR repair pathway for targeted mutagenesis, we used a similar approach as previously used in P. sojae. This implies cotransformations of three plasmids, i.e., two plasmids with sequences encoding Cas9 and gRNA, respectively, and the third one containing the HDR repair template (Fang & Tyler 2016).

For Avr1 we designed a construct to replace the coding region with HygB, a hygromycin-B resistance gene (Figure 1a). The inserts were flanked by a 1 kb right flanking arm and a 875 bp left flanking arm that are complementary to the target site for recombination. The reason for the shorter left flanking arm is a ~380 bp region in the genome assembly, that is not accessible for sequencing. We opted to use the largest possible flanking region, i.e. 875 bp (Figure 1a). For PiTubA2 we designed a construct to knock-in GFP just before the start codon of the open reading frame (Figure...
The repair template was designed such that the PAM would be disrupted upon HDR repair. For a number of organisms, higher efficiency of HDR repair has been reported when making use of single-stranded oligodeoxynucleotides (ssODN) as repair template, instead of plasmid DNA (Chen et al. 2011). We designed a ssODN repair template for AP5, comprising an 11 bp insert flanked by two 75 nt homology arms. The insert is designed in such a way that there is a stop codon (TAG) in each of the three open reading frames (Figure 1c). Further, the ssODN and gRNA were designed such that introduction of the 11 bp construct would disrupt the protospacer and consequently withhold Cas9 from further processing the target site. The expected protein product from the truncated gene contains the AP domain but lacks the GPCR domain.

Testing the detection limit

We next examined the sensitivity of our screening methods. To do so, we simulated the situation that an unknown fraction of transformants contains a Cas9-induced mutation. This is expected to be the case in a sample from pooled transformants after transformation; screening these for mutations can give an idea about the efficiency of targeted mutagenesis. First, we constructed an Avr1 amplicon (ΔAvr1) containing the 29 bp deletion that is expected after Cas9 cleavage at sites gRNA2 and gRNA3 (Figure S1a). Next, varying molar ratios of Avr1 and ΔAvr1 amplicons were annealed and incubated with T7 endonuclease I (T7EI), which is an enzyme that specifically digests mispaired DNA. We found that the detection limit is just over a molar ratio of 95:5 (i.e. 5% mutated amplicons) (Figure S1b).

In parallel, PCR amplicons were sequenced. When sequencing an amplicon that is obtained from a PCR on a pool of transformants of which a certain fraction un-
derwent CRISPR-induced mutagenesis, the resulting sequence chromatogram will contain a certain amount of ‘noise’, or background signals. These aberrant chromatogram peaks reflect the approximate frequency of amplicons with an indel, which can be estimated with an analysis tool such as TIDE (Brinkman et al. 2014). TIDE quantifies the editing efficacy by calculating the statistical probability of finding background signals in the chromatogram of a sample compared to finding them in a reference sequence chromatogram. A range of molar ratios of Avr1:∆Avr1 was sequenced, and by using T30-2 Avr1 as a reference, we analyzed the sequence chromatograms for mismatches. We found the lowest statistically significant detection at a molar ratio of 998:2 (i.e. 0.2% of the sequences having the deletion) (Figure S1c).

These detection limits are well below the observed frequencies of CRISPR-induced mutations in P. sojae, where up to 80% of the analyzed transformants have undergone NHEJ (Y.F. Fang, personal communication). Assuming that the CRISPR/Cas9 system is functional in P. infestans, our detection assays are sufficiently sensitive to reveal induced mutations. It should be noted however, that in the simulations described above only a single mutation (i.e. a 29 bp deletion) was present. In an actual sample of pooled transformed P. infestans protoplasts, a mix of differently sized indels is expected and the detection limit will be higher. Nonetheless, we assume that our detection assays are sufficiently sensitive to detect CRISPR-induced mutations in pooled P. infestans transformed protoplasts.

**In vitro activity assay**

To examine the functionality of the designed gRNAs, we performed an in vitro cleavage assay of target DNA. This assay can give an indication of the in vivo efficacy of the gRNA for Cas9 activity. We used purified Cas9 protein and in vitro transcribed gRNA in equimolar amounts, in combination with a PCR product as target DNA. We observed Cas9 activity on Avr1 using gRNA1 and gRNA3 (Figure 2a). In contrast, gRNA2 failed to direct Cas9 to the target site (Figure 2a). Examination of the sequence revealed a mistake in the design of the initial construct. When corrected, gRNA2 also showed activity (not shown). We did not observe Cas9 activity on PiTubA2 using gRNA8, nor on PiAP5 using gRNA183 (Figure 2b). In contrast to the initial gRNA2 on Avr1, we could not detect mistakes in the design of these two gRNAs. Consequently, judging from their inability to guide Cas9 in vitro, it is likely that also the in vivo efficacies of gRNA8 and gRNA183 are limited. Other gRNAs for these target genes may have an increased activity, but so far we have not tested alternative gRNAs for PiTubA2 and PiAP5.

**In vivo CRISPR/Cas9 in P. infestans**

Next, we set out to test the in vivo editing efficacy of the system. To this end, we first cloned the respective gRNAs into the expression plasmid pYF2.3-gRNA (Fang & Tyler 2016). We performed several transformations of P. infestans with plasmids pYF2.2-Cas9 and pYF2.3-gRNA and obtained numerous transformants resistant to the selection antibiotic geneticin. In these transformants, the presence of indels as a result of the presumed Cas9 activity was monitored by T7EI digestion and sequencing analysis. However, contrary to expectations set by the positive results of the use of CRISPR/Cas9 in P. sojae, P. capsici, and P. palmivora, the analyses did not reveal any lines in which the target gene was mutagenized. In some cases, the sequence chromatogram showed ambiguities near the target site, raising the hope that the mutagenesis was successful. More detailed analysis however, revealed that these lines were identical to the recipient isolate and should be considered as false-positives. Further trials focused at targeting Avr1 included transformations combining two or three gRNA-expressing vectors along with Cas9, but in all cases the results were negative.

In order to induce specific mutations in P. infestans, we performed experiments where plasmid DNA containing a HDR repair template for Avr1 was co-transformed with the CRISPR/Cas9 constructs. Even though several hygromycin-B resistant colonies were obtained, and the presence of HygB could be detected by PCR on genomic DNA, we were not able to detect integration of the transgene at the endogenous genomic locus. Possibly, the hygromycin-B resistance gene was integrated ectopically, or resistance was conferred by transcription from non-integrated plasmid DNA. Similarly, we did not obtain transformed lines with GFP-encoding sequences inserted at the 5’ end of PiTubA2, or PiAP5 mutants with the stop codon insertion.

For the target genes PiTubA1 and PiAP5 only a single gRNA was used. This and the fact the both gRNAs did not prove to be effective in the in vitro activity assay, are most likely the reasons for the absence of observable Cas9 activity. However, also in the case of Avr1 as target gene, where all three gRNAs proved functional in vitro, Cas9 activity appeared to be absent.

An obvious explanation for failure is poor expression of Cas9. To assess the expression level of Cas9, reverse-transcription PCR was performed on RNA isolated from mycelium of transformed lines. In several lines expression of Cas9 could be detected (Figure S2). Consequently, it is unlikely that lack of expression of Cas9 is the prime reason for failure of the system. As yet, we have not monitored the presence of the gRNA transcripts. Altogether, these findings led us to conclude that the CRISPR/Cas9 system in P. infestans is not
functioning, at least not up to the expected efficiency.

Modifications to the CRISPR/Cas9 system

It is hard to pinpoint the main cause for failure of the system, as multiple factors may have a role. Hence, we set out to make modifications at different steps in the process. Below we describe several modifications and alternative approaches in an effort to effectuate the CRISPR/Cas9 system in *P. infestans*.

Alternative vectors

In the original *P. sojae* CRISPR/Cas9 system, there is no selectable resistance marker present on the plasmid encoding the gRNA. However, in practice, co-transformations of plasmids usually result in integration of both plasmids (R. Weide, personal communication). Consequently, geneticin-resistant colonies are expected to also have incorporated the gRNA plasmid. Nevertheless, we decided to test whether we would be able to observe CRISPR/Cas9 activity by using a gRNA-encoding plasmid containing *HygB* as a resistance marker. To do so, we introduced the *HygB* in pYF2.3-gRNA-Ribo, replacing eGFP from the vector and yielding pJH2.4-gRNA-Ribo-Hyg (*Figure S3*). In this vector backbone, the respective gRNAs for the three target genes were cloned. Along with the Cas9 encoding plasmid, the resulting plasmids were used for cotransformations of *P. infestans*. Even though we obtained numerous hygromycin-B-resistant transformants, in none of the screened transformants we could detect mutagenized target genes.

In another attempt, we cloned the gRNA expression components in pYF2.2-Cas9, resulting in the ‘all-in-one’ vector pJH2.5-Cas9-gRNA (*Figure S3*). Numerous geneticin-resistant transformants were obtained and PCR analysis confirmed the presence of the Cas9 and gRNA genes. Unfortunately, no mutagenized target genes were observed. In addition, we constructed a vector containing both gRNA2 and gRNA3 (both targeting *Avr1*), but also here we did not obtain transformed lines with the expected deletion between the two target sites.

Nuclear localization sequence

Nuclear localization of Cas9 is essential. Oomycetes contain distinct nuclear localization sequence (NLS) signals, and commonly used mammalian NLS signals are not efficient in *P. sojae* (Fang & Tyler 2016; Fang et al. 2017). To overcome this, Fang & Tyler (2016) fused a synthetic NLS derived from a *P. sojae* bZIP transcription factor (TF) to Cas9. This NLS, further referred to as PsNLS, showed strong nuclear localization in *P. sojae* (Fang et al. 2017), and it is expected that it will perform likewise in *P. infestans*. To test the nuclear localization of Cas9, we obtained several transformed *P. infestans* lines carrying a PsNLS-Cas9-GFP fusion construct but unfortunately, for unknown reasons none of these lines showed fluorescence. Hence, we were unable to determine the localization of Cas9 in *P. infestans*. Anticipating that PsNLS is not able to target Cas9 to the nucleus in *P. infestans*, we set out to test a different NLS. Using a BLAST search, we identified the *P. infestans* homolog of the *P. sojae* bZIP TF, from which the NLS was obtained. This gene, PITG_11668, encodes a TF that was previously shown to have strong nuclear localization in *P. infestans* (Gamboa-Melendez et al. 2013). Next, we replaced the *P. sojae* NLS in vectors pYF2.3-PsNLS-Cas9 and pYF2.3-PsNLS-Cas9-GFP with the NLS region of PITG_11668 (PiNLS) to obtain the vectors pJH2.6-PiNLS-Cas9 and pJH2.6-PiNLS-Cas9-GFP, respectively (*Figure S3*). In addition, PiNLS was cloned N-terminally of GFP in the basic expression vector pGFP-N (Ah-Fong & Judelson 2011), to obtain pGFP-PiNLS-GFP (*Figure S3*). Next, these constructs were used for transformation of *P. infestans*. We performed cotransformations of pJH2.6-PiNLS-Cas9 along with

![Figure 2](https://example.com/figure2.png)

**Figure 2** | **In vivo cleavage assay.** a) gRNAs 1, 2, and 3 with *Avr1* as target DNA. b) gRNA8 with *PiTubA2* as target DNA (right) and gRNA183 with *PiAP5* as target DNA (left).
gRNAs for Avr1, PiTubA2, or PiAP5 carried by vectors pYF2.3-gRNA or pHJ2.4-gRNA-Hyg. Unfortunately, for none of the combinations we did obtain transformed lines in which we could observe Cas9 activity, nor did we obtain transformed lines exhibiting a fluorescent signal.

**gRNA expression**

In the *P. sojae* CRISPR/Cas9 system, expression of the ribozyme-gRNA construct as well as that of nptII, the gene providing geneticin resistance, is driven by pPsRPL41, the promoter of the *P. sojae* ribosomal gene PsRPL41. Thus, geneticin-resistant transformants should in principle express the ribozyme-gRNA construct. However, in the numerous geneticin-resistant transformants that we have obtained, we did not observe CRISPR/Cas9 activity. Consequently, we questioned whether the promoter of PsRPL41 is active in *P. infestans*. In an earlier study performed in *P. infestans*, the expression stability of the *P. infestans* ortholog of pSrPL41 was found to be inferior to other *P. infestans* and *P. capsici* ribosomal promoters (Poidevin et al. 2015). pSrPL41 was not evaluated. To assess whether the use of a different promoter driving the expression of the ribozyme-gRNA construct could improve CRISPR/Cas9 activity, we opted to use the *P. capsici* S9 promoter (pPsS9). This promoter was found to provide high and stable expression in *P. infestans* (Poidevin et al. 2015). To replace pSrPL41 with pPsS9, the promoter was PCR amplified from pTOR-S9 (Poidevin et al. 2015) and cloned into EcoRI/Nhel sites of pYF2.3-gRNA-Ribo. However, an unexpected Nhel site at approximately 150 bp upstream of the 3’ end of the promoter sequence, gave rise to a truncated insert, as the same restriction enzyme is used for gRNA insertion. To circumvent this problem pPsS9 and the gRNA can be introduced into the vector backbone by Gibson assembly but due to time constraints we have not been able to obtain a suitable construct with pPsS9 for transformation of *P. infestans*.

**Delivery of ribonucleoprotein (RNP) complexes**

In several organisms it has been shown that the delivery of Cas9 protein-gRNA ribonucleoproteins (RNP) complexes is an efficient method for inducing targeted genome editing events, including a number of plant species (Woo et al. 2015), the nematode *Caenorhabditis elegans* (Cho et al. 2013), filamentous fungi and yeast (Pohl et al. 2016; Grahl et al. 2017), protozoa (Soares Medeiros et al. 2017), and also in human cell lines (Ramakrishna et al. 2014). Moreover, it was found to result in genome editing with substantially higher specificity compared to DNA transformation (Zuris et al. 2015). From a technical perspective, the use of RNP complexes may have several advantages over a ‘regular’ CRISPR system where both components are delivered as plasmid DNA and integrated into the genome. Firstly, the genome-editing efficiency of Cas9 using RNP complexes does not rely on transcription rate in the host, as both components are preassembled in vitro prior to delivery into cells. In *Phytophthora* spp., integration of foreign DNA occurs randomly, and it is plausible that integration in a ‘silent’ part of the genome results in reduced transcription rates. The integration can also lead to disruption of genes, when the transgene is inserted in an open reading frame. Secondly, and more importantly, transgene expression of Cas9 can have adverse effects and can even lead to cell death due to toxicity (Jiang et al. 2014; Kim et al. 2014; Peng et al. 2014). Generating CRISPR-induced mutations using RNP complexes might (partially) alleviate this issue, as after some time the complexes are degraded, reducing chances for side effects.

To our knowledge, the use of RNP complexes for targeted genome editing has not been explored in oomycetes. To assess whether RNP complexes can be used for targeted genome editing in *P. infestans*, we used a modified protocol for PEG-mediated protoplast transformation, substituting plasmid DNA with pre-assembled RNP complexes. In parallel, one sample was co-transfected with the Avr1 HDR construct (Figure 1a), which has HygB as a selectable marker. After transfection, regenerated protoplasts were analyzed by a T7EI assay and sequencing. These analyses showed all samples to be identical to control transformants, with no significantly overrepresented aberrant background signals in the sequence chromatograms. Moreover, we could not confirm the integration of HygB at the target locus, not even in transformants that were hygromycin-B resistant.

The experimental setup of direct delivery of RNP complexes has some limitations, most importantly the lack of selection. Whereas in a regular plasmid-based transformation experiment the introduced plasmid contains a resistance gene to a selection antibiotic, protein transfection does not yield antibiotic resistant colonies and hence also non-transfected protoplasts will regenerate. Consequently, in our experiments transfected protoplasts might have been overshadowed by non-transfected protoplasts. Moreover, despite careful preparation of the starting material, some residual sporangia may have been present in the protoplast suspension and these also readily overgrow regenerating protoplasts.

Another potential limitation is the nuclear localization of the RNP complexes. The Cas9 protein used in this experiment contains two Simian virus 40 (SV40) T antigen NLS tags, one at the N-terminus and the other at the C-terminus. The SV40 NLS is a well-studied
monopartite NLS tag consisting of several basic amino acids (Lange et al. 2007), and has been shown to localize fusion gene products to the nucleus of *P. sojae*, albeit with a reduced efficiency compared to other NLS tags (Fang et al. 2017). Hence, it is expected that the RNP complexes used in this study are localized to the nucleus. Still, the use of a Cas9 protein equipped with a *Phytophthora* specific NLS tag might increase the efficiency (Fang et al. 2017). Since such Cas9 proteins are not commercially available they would have to be produced in-house.

**Conclusions and future outlook**

Contrary to expectations set by the successful application of the CRISPR/Cas9 system for targeted genome editing in *P. sojae*, *P. capsici* (B.M. Tyler, personal communication), and *P. palmivora* (Gumtow et al. 2017), we have as yet not been able to implement the system in *P. infestans*. The same holds for colleagues elsewhere who are also experiencing difficulties in effectuating the CRISPR/Cas9 system for use in *P. infestans* (personal communication). It is, however, hard to pinpoint the cause for failure of the system. Likely, it is an additive effect of several suboptimal conditions, such as Cas9 or gRNA expression levels, Cas9 localization, or the incubation temperature. Fang and Tyler (2016) who established the system in *P. sojae* also faced numerous challenges and made substantial modifications in the initial CRISPR/Cas9 procedure to get it to work in *P. sojae*. For *P. palmivora* Gumtow et al. (2017) choose Agrobacterium-mediated transformation (AMT) to implement the CRISPR/Cas9 system. Whereas protoplast transformation typically results in multiple integration events of the transgene(s), AMT usually gives rise to only one or two integrations of the transgene in the genome (Vijn & Govers 2003). A higher transgene copy number easily results in altered expression levels due to homology-dependent gene silencing or overexpression of the transgene. However, *P. infestans* transformants resulting from AMT rarely show silencing or overexpression of the target gene (B.I. van de Vondervoort, unpublished data), a phenomenon that might be due to the low integration rate. Overall, generating transformants via AMT is a phenomenon that might be due to the low integration rate. Overall, generating transformants via AMT is rarely show silencing or overexpression of the transgene. A staggered DSB break with a 4 or 5 nt 5' overhang (Zetsche et al. 2015). These cohesive ('sticky') ends can increase the frequency of HDR repair. Moreover, on certain positions, Cas12a is sensitive to single-base mismatches in the protospacer, reducing the frequency of off-target cleavage (Kleinistiver et al. 2016). Also, its T-rich PAM (TTTN) may limit the number of target sites in *P. infestans*, which has a GC content of 51% (Haas et al. 2009).

We trust that, with dedicated effort, developing a CRISPR/Cas system for *P. infestans* is attainable. Future work should focus on systematic analysis of factors limiting the efficiency of the system. When these
limitations are identified and overcome, targeted mutagenesis in \( P. \) infestans might be within reach.

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Materials and methods

Strains, culture conditions and transformations

\( P. \) infestans strain T30-2 (van der Lee et al. 2001) and all transgenic lines were routinely grown at 18 °C in the dark on rye agar medium supplemented with 2% sucrose (RSA) (Caten & Jinks 1968). RSA was supplemented with 20 µg/mL vancomycin, 100 µg/mL ampicillin and 50 µg/mL amphotericin B, and in addition, for transformed lines with 2.5 µg/mL G418.Transient and stable transformants of \( P. \) infestans were generated using PEG/CaCl2-mediated protoplast transformation and zoospore electroporation. Protoplast transformation was performed according to methods described previously (Ah-Fong et al. 2008), omitting the step of complexing circular plasmid DNA with Lipofectin in protoplast transformation. Zoospore electroporation was performed following available protocols.

gRNA design

Sequences for target genes were downloaded from FungiDB (Stajich et al. 2012). The full-length gene products were PCR amplified and subsequently validated by sequencing (Eurofins, Ebersberg, Germany). Next, the sequence was screened for CRISPR sites using NGS as PAM on Geneious R9.1.4 (Kearse et al. 2012). The resulting sites were scored for on-target activity according to Doench et al. (Doench et al. 2014) and for off-target interactions according to Hsu et al. (2013). The top 10 best scoring CRISPR sites were manually curated using BLAST analysis on the \( P. \) infestans reference genome and predictions of secondary RNA structure of the corresponding gRNAs using RNAstructure (Reuter & Mathews 2010).

gRNAs for Avr1 and PiTubA1 were ordered as sense and antisense PAGE-purified oligos (Integrated DNA Technologies) and subsequently annealed and ligated into pYP2.3-gRNA-Ribo-EV, as described by Fang and Tyler (2016), using Bsal and Nhel-HF (New England Biolabs). For \( PiAP5 \), 283 bp constructs transcribing the ribozyme-gRNA insert flanked by two 30 bp overlaps, were designed and ordered as gBlocks (Integrated DNA Technologies). Next, the fragments were inserted into pYP2.3-gRNA-Ribo-EV by Gibson assembly using the NEBuilder HiFi kit (New England Biolabs Inc).

Constructs

Figure S3 shows the constructs used in this study. The hygromycin B resistance gene was amplified from pGFp-H (Ah-Fong & Judelson 2011) using primers Hyg_AflII_F and Hyg_Apal_R, and the resulting amplicon was cloned into pYP2.3-gRNA-Ribo using AflII and Apal (New England Biolabs), yielding pJH2.4-Hyg-gRNA. To constitute the 'all-in-one' vector pH2.5-Cas9-gRNA, the gRNA expressing construct from pYP2.3-gRNA (containing the RPL41 promoter, gRNA insert, and Hsp70 terminator) was inserted into pH2.2-Cas9 using EcoRI (Promega). A dual-gRNA vector encoding Avr1-targeting gRNA2 and gRNA3 was constructed by inserting the ribozyme-gRNA3 construct into pH2.2-gRNA2 by Gibson assembly. The amplification containing the insert was made using primers gRNA3_Gibson_F and gRNA3_Gibson_R. The NLS region from PiTG_11668 was inserted at the N-terminus of GFP. The PCR amplicon was cloned into the AgeI and Nhel sites of pGFp-N (Ah-Fong & Judelson 2011), yielding pH2.6-PiNLS-Cas9 and pH2.6-PiNLS-Cas9-GFP. The NLS region from PiTG_11668 was inserted into the SacII and SpeI sites of pH2.2-PiNLS-Cas9 and pH2.2-PiNLS-Cas9-GFP. \( \Delta \)Avr1 was constructed by overlap extension PCR using primers Avr1_del_F and Avr1_del_R. All primers used in this study are listed in Table S1.

Cas9 in vitro activity assay

To obtain template DNA for in vitro transcription (IVT), a PCR was performed with a T7 promoter-fused forward primer (marked with extension _pT7_F) and the primer sgRNA_Col_R, using plasmid DNA containing the respective gRNAs as template. Next, gRNA was transcribed with T7 RNA polymerase using MEGashortscript T7 kit (Thermo Fischer Scientific). IVT was allowed to proceed for 4 h, after which RNA was purified by phenol/chloroform and ethanol precipitation, and analyzed on agarose gel. Target DNA was amplified using the respective primers for full-length PCR products of Avr1, PiTubA2, and PiAP5. SpCas9 nuclease was purchased (New England Biolabs). The assay was performed according to manufacturer's instructions.

Molecular analysis of transformants

Genomic DNA (gDNA) was extracted from pooled or individual \( P. \) infestans transformants according to methods described previously (Fang & Tyler 2016), with modifications. Pooled transformants, 24-48 h after transformation, were pelleted by centrifugation, resuspended in 500 mL of gDNA extraction buffer (200 mM Tris, pH 8.0, 200 mM NaCl, 25 mM EDTA, pH 8.0, 2% SDS, plus 0.1 mg/mL RNase A added prior to use) and sheared by vigorous pipetting. For individual transformants, approximately 250 µL of \( P. \) infestans mycelium was frozen in liquid nitrogen, freeze-dried, and ground to a powder using a Retsch Mixer Mill MM 400 and metal beads (ø 3 mm) for 30 seconds at 30 Hz. Subsequently, the powder was resuspended in 500 µL of gDNA extraction buffer. DNA was recovered by phenol/chloroform extraction and isopropanol precipitation. RNA was isolated using home-made TRIzol (Verdonk 2014), and cDNA was synthesized using M-MLV Reverse Transcriptase (Promega) according to manufacturer's instructions. RT-PCR was performed using primers Cas9_RT_F and Cas9_RT_R.

All PCR amplifications were conducted using Q5 high-fidelity DNA polymerase (New England Biolabs). Nested PCR was performed using diluted (1000x) PCR products as a DNA template. PCR amplifications were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Sequence analysis was performed at Eurofins.
T7EI assay

The efficiency of the CRISPR/Cas9 system was tested with T7 endonuclease I (T7EI) (New England Biolabs), using gDNA isolated from pooled transformants as template. First, the target gene was amplified from gDNA using primers amplifying the full-length sequence of the respective target genes (Table S1). Next, purified amplicons were annealed in a thermocycler with the following conditions: 95°C for 5 min, ramp down to 85°C at -2°C/s, ramp down to 20°C at -0.1°C/s, hold at room temperature. The T7EI cleavage assay was performed according to manufacturer’s instructions and analyzed by gel electrophoresis.

RNP assembly and transfection

Ribonucleoprotein complexes (RNPs) of Cas9 (EnGen Cas9 NLS, New England Biolabs) and gRNAs were prepared immediately before transfections. After IVT, gRNAs were refolded by heating at 90°C for 5 min and cooling to room temperature over the course of 12 h. To preassemble RNPs, equimolar amounts of Cas9 (120 pmol) and 1 M mannitol, without antibiotics) and protoplasts were regener-

References


function, and interaction with importin alpha, \textit{J Biol Chem} 282: 5101-5105. doi: 10.1074/jbc.R60026200
Supplementary files

Figure S1 | Detection limit assays. a) PCR amplicons used for the detection limit assays. ΔAvr1 contains a 29 bp deletion. b) T7EI assay. Varying amounts of Avr1 and ΔAvr1 PCR amplicons were annealed and digested by T7EI. c) Example output from TIDE, confirming the presence of the 29 bp deletion in ΔAvr1 (left red bar). Here, a sequence chromatogram obtained from sequencing a molar ratio of 998:2 Avr1:ΔAvr1 was compared to a sequence chromatogram of Avr1. The predicted frequency of chromatograms with a deletion (1.5%) deviates from the actual molar ratio of Avr1:ΔAvr1 (0.2%).

Figure S2 | Expression analysis of Cas9 in 11 selected transformed lines. cDNA and genomic DNA (gDNA) of *P. infestans* strain T30-2 were used as negative control and gDNA from a *P. infestans* Cas9 transformant as positive control.
Figure S3 | Plasmids used in this study. Plasmids starting with ‘pYF’ are obtained from Francis Fung (Fang & Tyler 2017), plasmids starting with ‘pJH’ are modified versions as described in this report.
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