Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) **17**(1), 127–139 DOI: 10.1111/mpp.12318

Technical advance

Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9

YUFENG FANG[1,2] AND BRETT M. TYLER[1,2,*]

[1]*Interdisciplinary PhD Program in Genetics, Bioinformatics & Computational Biology, Virginia Tech, Blacksburg, VA 24061, USA*
[2]*Center for Genome Research and Biocomputing and Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA*

SUMMARY

Phytophthora sojae is an oomycete pathogen of soybean. As a result of its economic importance, *P. sojae* has become a model for the study of oomycete genetics, physiology and pathology. The lack of efficient techniques for targeted mutagenesis and gene replacement have long hampered genetic studies of pathogenicity in *Phytophthora* species. Here, we describe a CRISPR/Cas9 system enabling rapid and efficient genome editing in *P. sojae*. Using the RXLR effector gene *Avr4/6* as a target, we observed that, in the absence of a homologous template, the repair of Cas9-induced DNA double-strand breaks (DSBs) in *P. sojae* was mediated by non-homologous end-joining (NHEJ), primarily resulting in short indels. Most mutants were homozygous, presumably as a result of gene conversion triggered by Cas9-mediated cleavage of non-mutant alleles. When donor DNA was present, homology-directed repair (HDR) was observed, which resulted in the replacement of *Avr4/6* with the *NPT II* gene. By testing the specific virulence of several NHEJ mutants and HDR-mediated gene replacements in soybean, we have validated the contribution of Avr4/6 to recognition by soybean *R* gene loci, *Rps*4 and *Rps*6, but also uncovered additional contributions to resistance by these two loci. Our results establish a powerful tool for the study of functional genomics in *Phytophthora*, which provides new avenues for better control of this pathogen.

Keywords: Avr4/6, CRISPR/Cas9, gene replacement, genome editing, oomycetes, *Phytophthora sojae*, RXLR effector.

INTRODUCTION

Phytophthora sojae causes 'damping off' of soybean seedlings as well as stem and root rot of established plants (Tyler, 2007). Morphologically and physiologically, oomycetes such as *P. sojae* resemble filamentous fungi, but evolutionarily they are classified in the kingdom Stramenopila (Tyler, 2001). Most *Phytophthora* species are plant pathogens that can damage a huge range of agriculturally and ornamentally important plants (Erwin and Ribeiro, 1996). Because of its economic impact, *P. sojae*, together with *P. infestans*, has been developed as a model species for the study of oomycete plant pathogens (Tyler, 2007).

The first two genome sequences of oomycetes (*P. sojae* and *P. ramorum*) were published approximately 9 years ago (Tyler *et al*., 2006), but functional genomics studies have been hampered by the lack of efficient strategies for genome engineering. DNA transformation procedures have been developed (Judelson *et al*., 1993a,b), but gene knockouts and gene replacements have never been possible because insertion of transgenes occurs exclusively by non-homologous end-joining (NHEJ) (Judelson, 1997; Tyler and Gijzen, 2014). Alternative approaches for functional analysis have included TILLING (Targeting Induced Local Lesions in Genomes) (Lamour *et al*., 2006) and gene silencing (Ah-Fong *et al*., 2008; Judelson *et al*., 1993b; Wang *et al*., 2011; Whisson *et al*., 2005). However, TILLING, which is based on random mutagenesis, is very laborious, requires long-term storage of large pools of mutants and has not been proven to be very useful in oomycetes. Gene silencing (RNAi, RNA interference), triggered using hairpin, antisense and sense RNA constructs (Ah-Fong *et al*., 2008) or using double-stranded RNA (dsRNA) directly (Wang *et al*., 2011; Whisson *et al*., 2005), has been proven to be useful. However, knockdown of genes in oomycetes by RNAi is incomplete, and varies among gene targets, experiments and laboratories. Also, selective silencing of closely related genes is difficult.

Recent advances in engineered nucleases that specifically cleave genomic sequences in living cells have provided valuable tools to create targeted mutations in numerous organisms, from vertebrates, insects and plants (reviewed in Gaj *et al*., 2013) to microbes, including parasites (Peng *et al*., 2015; Shen *et al*., 2014; Wagner *et al*., 2014; Zhang *et al*., 2014) and fungi (Jacobs *et al*., 2014; Liu *et al*., 2015; Vyas *et al*., 2015). These nucleases, which include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated), can generate a double-strand break (DSB) at specific sites. By trigger-

*Correspondence: Email: Brett.Tyler@oregonstate.edu

ing repair of the DSB, by either error-prone NHEJ or homology-directed repair (HDR), such methods can increase the rate of gene editing to levels that enable the ready isolation of cells or organisms bearing a desired genetic change (Miller *et al*., 2011). ZFNs and TALENs are engineered proteins containing a modular DNA recognition domain and a DNA cleavage domain.

Like ZFNs and TALENs, the type II CRISPR/Cas9 system derived from the adaptive immune system of *Streptococcus pyogenes* also has DNA recognition and cleavage functions (Cong *et al*., 2013; Mali *et al*., 2013). However, DNA recognition is mediated by a single guide RNA (sgRNA) rather than a fused DNA recognition protein domain. The specificity of this system relies on the sgRNA, which can direct the nuclease Cas9 to the target DNA sequence (Cong *et al*., 2013; Mali *et al*., 2013).

Here, we have implemented the CRISPR/Cas9 system in *P. sojae*, using the RXLR effector gene *Avr4/6* (Dou *et al*., 2010) as a target. RXLR effectors are a large superfamily of virulence proteins secreted by many oomycetes that have the ability to enter host cells in order to promote host susceptibility (Jiang and Tyler, 2012). The presence of some RXLR effectors, such as Avr4/6, can be recognized by intracellular receptors encoded by plant resistance genes (*R* genes), triggering vigorous defence responses (Jiang and Tyler, 2012). The presence of Avr4/6 is recognized by soybean *R* genes *Rps*4 and *Rps*6 (Dou *et al*., 2010; Gijzen *et al*., 1996; Whisson *et al*., 1994); recognition by *Rps*4 requires the N-terminus of Avr4/6, whereas recognition by *Rps*6 requires the C-terminus (Dou *et al*., 2010). Our results demonstrate that CRISPR/Cas9-mediated gene disruption and gene replacement is an efficient and useful strategy for the testing of the function of specific genes in *P. sojae*, such as *Avr4/6*, which should be useful for all oomycetes.

RESULTS

Establishment of the CRISPR/Cas9 system for *P. sojae*

To establish a CRISPR/Cas9 system for *P. sojae*, we had to establish efficient expression of Cas9, efficient expression of guide RNAs and targeting of the Cas9 enzyme to the nucleus.

For expression in *P. sojae*, we selected the *Streptococcus pyogenes* Cas9 encoded by a gene with human-optimized codons (*hSpCas9*), because this Cas9 version has been used in diverse organisms (Cong *et al*., 2013; Peng *et al*., 2015; Zhang *et al*., 2014) and matches *P. sojae* codon usage relatively well. To test whether this protein could be efficiently expressed in *P. sojae*, we fused green fluorescent protein (GFP) to the C-terminus of hSpCas9. Furthermore, to direct hSpCas9-GFP into the *P. sojae* nucleus, we used a strong synthetic nuclear localization sequence (NLS) derived from a *P. sojae* bZIP transcription factor (Fang and Tyler, 2015), which we fused to the N-terminus of SpCas9 (Fig. 1A). Preliminary experiments had shown that commonly used mammalian NLS signals did not work efficiently in *P. sojae*. Expression of the *P. sojae* NLS (PsNLS) fused hSpCas9-GFP construct in *P. sojae* transformants resulted in a bright GFP signal strongly localized within the nuclei of *P. sojae* hyphae (Fig. 1A). These results indicated that hSpCas9 was strongly expressed in *P. sojae* without further codon optimization, and that the bZIP-derived NLS efficiently targeted the fusion protein to *P. sojae* nuclei.

In most systems, sgRNAs are synthesized by RNA polymerase III (RNA pol III), typically using a U6 small nuclear RNA (snRNA) promoter (Cong *et al*., 2013; Hwang *et al*., 2013; Mali *et al*., 2013; Shen *et al*., 2014; Zhang *et al*., 2014). However, no RNA pol III promoters have yet been functionally defined in oomycetes. U6 gene sequences are highly conserved among different oomycetes (Fig. S1A, see Supporting Information), and so we cloned the full-length *P. sojae* and *P. infestans* U6 gene regions (Fig. S1B,C) and inserted a 150-bp fragment of the *eGFP* (*enhanced green fluorescent protein*) gene near the 3' end as a polymerase chain reaction (PCR) reporter sequence (Fig. S1D). Surprisingly, however, we did not detect any transcripts spanning the GFP reporter fragment by reverse transcription-polymerase chain reaction (RT-PCR) (data not shown).

Recently, the generation of sgRNAs from RNA pol II promoters has been demonstrated in wheat (Upadhyay *et al*., 2013), yeast (Gao and Zhao, 2014) and *Arabidopsis* (Gao *et al*., 2015). The yeast and *Arabidopsis* systems used *cis*-acting ribozymes to trim flanking sequences from the sgRNAs, whereas the wheat system did not. Thus, we employed the constitutive *P. sojae RPL41* promoter (Dou *et al*., 2008a,b) to direct the transcription of sgRNAs, and evaluated sgRNA constructs that either were or were not flanked on the 5' side by a hammerhead (HH) ribozyme and on the 3' side by a HDV ribozyme (Gao and Zhao, 2014) (Fig. 1B).

To simplify the generation and screening of *P. sojae* transformants, the hSpCas9 gene and a resistance selection marker (*NPT II*) were placed in one plasmid, whereas the sgRNA gene together with a GFP marker gene were placed in a second plasmid (Figs 1B and S4, see Supporting Information).

Cas9-mediated mutagenesis of *Avr4/6*

To test the *P. sojae* sgRNA:Cas9 system, we selected as a target a *P. sojae* gene encoding an RXLR avirulence effector, Avr4/6 (GenBank: GU214064.1). *Avr4/6* is a single copy gene with no close paralogues. Furthermore, loss of *Avr4/6* function was expected to confer a phenotype that would not affect *in vitro* growth, namely the ability to successfully infect soybean cultivars containing resistance genes *Rps*4 or *Rps*6 (Dou *et al*., 2010). sgRNAs targeting *Avr4/6* were designed using the web tool *sgRNA Designer* (Doench *et al*., 2014). sgRNA candidates rated highly by the tool were further filtered by off-target analysis. Finally, two sgRNAs (sgRNA versions A and B) were selected in which the

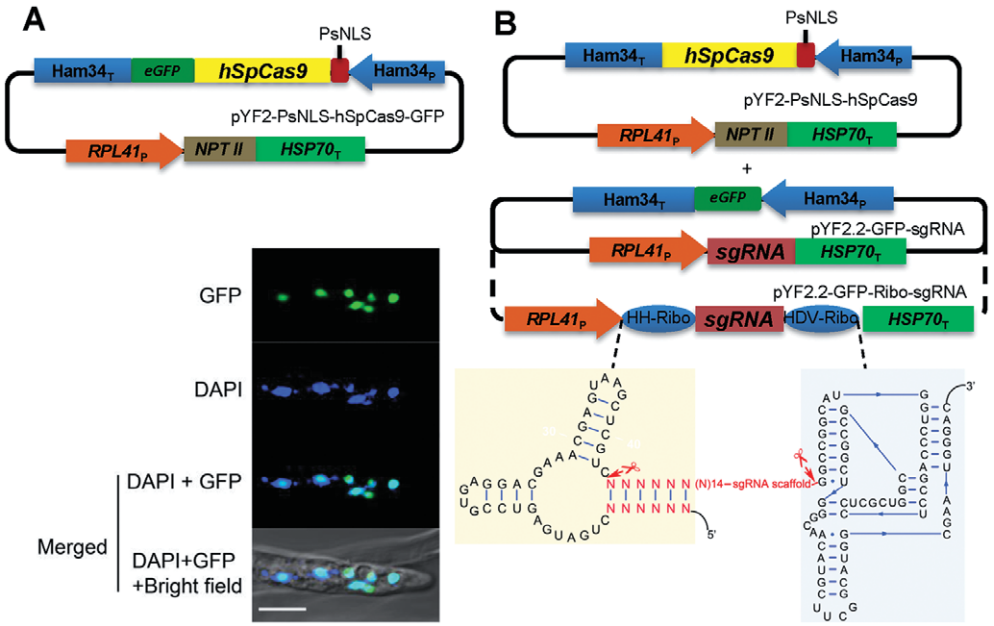


Fig. 1 Cas9 and guide RNA constructs for *Phytophthora sojae* genome editing. (A) Top: plasmid for expression of hSpCas9 fused to eGFP and a nuclear localization sequence (NLS) in *P. sojae*. PsNLS, a strong synthetic NLS derived from a *P. sojae* bZIP transcription factor; eGFP, enhanced green fluorescent protein. Bottom: *P. sojae* hyphae expressing PsNLS-hSpCas9-GFP from pYF2, counter-stained with 4',6-diamidino-2-phenylindole (DAPI); scale bars, 10 μm. (B) Top: plasmids for expression of CRISPR constructs in *P. sojae*. Cas9 expression is driven by the Ham34 promoter, on a plasmid with the selectable marker *NPT II* driven by the *P. sojae RPL41* promoter. Transcription of single guide RNA (sgRNA) (including flanking ribozymes) is driven by the *RPL41* promoter on a plasmid with an eGFP expression cassette (used as a screening marker). Bottom: double ribozyme construct for release of sgRNAs from the primary RNA polymerase II transcript.

respective Cas9 cleavage sites overlapped unique restriction enzyme sites (*Bst* UI and *Tsp* 45I, respectively) that could be used to rapidly screen for mutations (Fig. 2A).

To examine the activity of the designed sgRNAs, Avr4/6-A and Avr4/6-B, we carried out sgRNA-mediated *in vitro* cleavage assay of target DNA. In these assays, the sgRNAs were synthesized using T7 RNA polymerase and purchased SpCas9 protein. Cas9/sgRNA-B completely cleaved the target DNA, whereas Cas9/sgRNA-A showed no activity *in vitro* (Fig. 2B).

In parallel with the *in vitro* assays, we used transient expression in *P. sojae* protoplasts to test whether the hSpCas9 and sgRNAs produced *in vivo* could modify the endogenous *Avr4/6* gene. The two *Avr4/6*-specific sgRNAs were assembled into the *P. sojae* expression plasmid under the control of the *RPL41* promoter, either flanked with (Avr4/6-sgRNA-A_R, -B_R) or without (Avr4/6-sgRNA-A, -B) ribozymes. The sgRNA constructs were co-transformed with the hSpCas9 expression plasmid into *P. sojae* strain P6497. Transformants were enriched by G418 selection 12 h after transformation when hyphae had regenerated. After 24 h, DNA was extracted from the culture containing the pooled transformants. *Avr4/6* sequences were amplified from the pool of genomic DNAs (gDNAs) and screened for mutants resistant to the relevant restriction enzymes (*Bst* UI for A and *Tsp* 45I for B). We found that the *Avr4/6* amplicons from the two sgRNA-A transformations (constructs with and without ribozymes) were still fully subject to restriction enzyme cleavage, indicating failure of Cas9-mediated mutagenesis (Fig. 2C). In contrast, the transformation utilizing sgRNA version B flanked by ribozymes yielded restriction enzyme-resistant amplicons (Fig. 2C). However, the transformation utilizing sgRNA version B without ribozymes did not yield restriction enzyme-resistant amplicons (Fig. 2C). To validate the enzyme cleavage results, we sequenced the nested PCR products amplified from the enzyme digestion products from the sgRNA-B_R transformants. The sequence chromatograms showed pure sequences proximal to the target site and mixed sequences distal to the target site (data not shown), suggesting the presence of mutations at the target site. These observations indicate that, in *P. sojae*, RNA pol II can be successfully used for the generation of sgRNA, provided that ribozymes are employed to remove the surrounding sequences from the transcripts. The failure of sgRNA version A may result from strong self-complementarity that was subsequently discovered in its sequence, which could block its binding to target DNA.

To characterize CRISPR/Cas9-generated *Avr4/6* mutations in detail, the transformation with the ribozyme-containing Avr4/6-sgRNA-B_R construct was repeated. Individual G418-resistant transformants were isolated and screened for the presence of GFP, indicating the presence of the sgRNA construct. Of 50 primary transformants screened, six exhibited green fluorescence. Of these, four yielded *Avr4/6* amplicons that were partially or fully resistant to *Tsp* 45I digestion (Fig. 3A), indicating the presence of

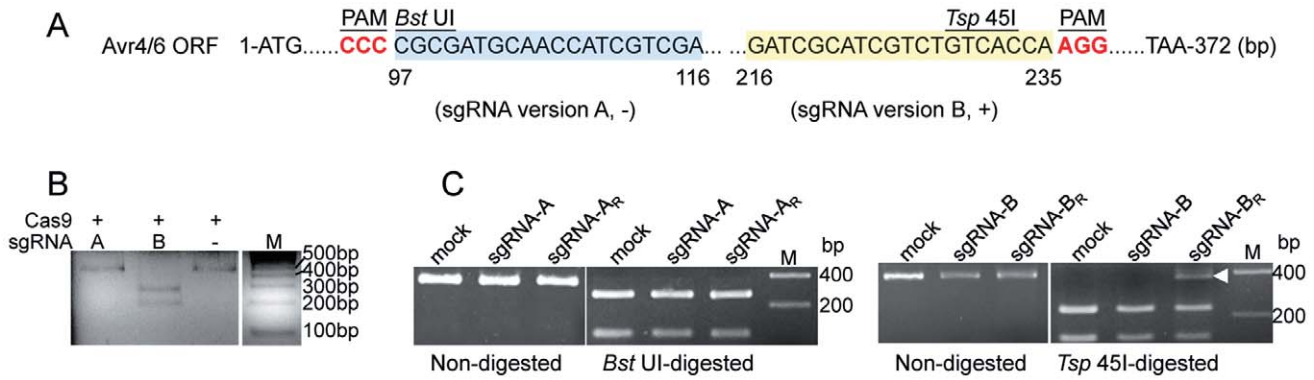
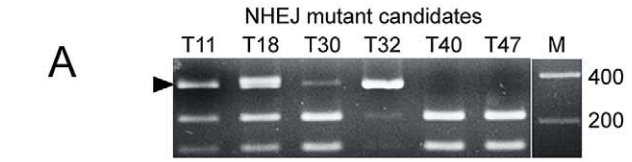


Fig. 2 Single guide RNAs (sgRNAs) for targeting of *Avr4/6*. (A) Two sgRNA target sites within the *Avr4/6* open reading frame (ORF). Target sites of sgRNA A (sgRNA-A) and B (sgRNA-B) are highlighted in blue and yellow, respectively. sgRNAs A and B target *Avr4/6* on the negative (–) and positive (+) DNA strand, respectively. The sgRNA-A site overlaps with a *Bst* UI restriction enzyme site (CGCG) and sgRNA-B with a *Tsp* 45I site ($GT^{G}/_{C}AC$). PAM, Protospacer Adjacent Motif (bold red). (B) *In vitro* cleavage assay indicating that Avr4/6 sgRNA-B can direct Cas9 cleavage of target polymerase chain reaction (PCR) products, but sgRNA-A cannot. The DNA template was amplified from pBS-Avr4/6 using M13F and M13R [supplemental sequences in Appendix S1 (see Supporting Information)]. The colours of the original gel are inverted for clarity. (C) PCR and restriction enzyme analysis of *Phytophthora sojae* pooled transient expression transformants, indicating that only sgRNA-B flanked by ribozymes (sgRNA-B_R) produced amplicons resistant to restriction enzyme cleavage (arrowhead). Approximately 25%–30% of the amplicon was resistant to *Tsp* 45I digestion. The experiment was performed in triplicate. sgRNA-A, -B, sgRNA lacking ribozymes; sgRNA-AR, -BR, sgRNAs flanked byribozymes; mock, *P. sojae* transformants only receiving Cas9 plasmid. In (B) and (C), all gel panels placed together were from the same gel; white dividers indicate lanes not adjacent in these gels.



B

Tsp 45I PAM

```
Avr4/6   GCTGCGATCGCATCGTCTGTCA CCAAGGAAGAAGAGGAACGC
           A  A  I  A  S  S  V   T  K  E  E  E  E  R
T11-1    GCTGCGATCGCATCG------A CCAAGGAAGAAGAGGAACGC
           A  A  I  A  S         T  K  E  E  E  E  R
T11-2-5  GCTGCGATCGCATCGTCTGTC- CCAAGGAAGAAGAGGAACGC
           A  A  I  A  S  S  V    P  R  K  K  R  N
T18-1    GCTGCGATCGCATCGTCTGT-- -aAAGGaagaagaggaacgc
           A  A  I  A  S  S  V      K  E  E  E  E  R
T18-2    GCTGCGATCGCATCGTCTGT-- -CAAGGAAGAAGAGGAACGC
           A  A  I  A  S  S  V      K  E  E  E  E  R
T32-3    GCTGCGATCGCATCGTCTGTCAaCCAAGGAAGAAGAGGAACGC
           A  A  I  A  S  S  V  N  Q  G  R  R  G  T
```

	Williams			Harosoy		
Mut type	*rps*	*Rps4*	*Rps6*	*rps*	*Rps4*	*Rps6*
Wild type	V	A	A	V	A	A
In-frame deletion	V	I/V	I/A	V	I/V	I/A
Deletion, frame shifted	V	A	I/A	V	A	I/A
In-frame del and C>A (rare codon mutation)	V	I	A	V	A	A
In-frame deletion	V	I/A	A	V	A	I/A
Insertion, frame shifted	V	I/V	I/A	V	I	I

Fig. 3 Characterization of individual non-homologous end-joining (NHEJ)-mediated mutants. (A) *Tsp* 45I screening of *Avr4/6* amplicons from six individual transformants carrying hSpCas9 and sgRNA-B_R plasmids. Arrowhead indicates *Tsp* 45I-resistant amplicons. Transformants are expected to carry a mixture of modified and non-modified *Avr4/6* genes. (B) Left: sequences of *Avr4/6* mutant amplicons from single zoospore lines derived from transformants T11, T18 and T32 (T30 produced no zoospores). Target sites are highlighted in yellow and the PAM sequences are shown in bold red. Right: summary of virulence assays of *Avr4/6* mutants on *Rps*4- and *Rps*6-containing cultivars. V, virulent; A, avirulent; I, intermediate; I/A intermediate to avirulent; I/V intermediate to virulent.

Table 1 Avr4/6 CRISPR/Cas-induced non-homologous end-joining (NHEJ) mutations.

	Mutant patterns					
	Homozygotes			Heterozygotes		Biallele
Mutants	WT/WT*	mut1/mut1	mut2/mut2	WT/mut1	WT/mut2	mut1/mut2,3
T11†	0	2	5	0	0	3‡
T18§	0	9	1	0	0	0
T32¶	0	10	0	0	0	0

*WT, wild-type sequence.

†T11, mut1, 6-bp deletion; mut2, 1-bp insertion.

‡Heterozygous mut1/mut3; mut3 is a 3-bp deletion. The sequencing profiles of the heterozygotes were disambiguated using the web-tool TIDE (Brinkman *et al*., 2014).

§T18, mut1, 3-bp deletion; mut2, 3-bp deletion plus 1-bp substitution.

¶T32, mut1, 1-bp insertion.

Avr4/6 mutations. As *P. sojae* protoplasts and hyphae are multinucleate, and hence might be expected to harbour nuclei with a diversity of *Avr4/6* mutations, we isolated zoospores (which are mononucleate) from three of the primary transformants (T11, T18 and T32; the fourth, T30, did not produce zoospores). Ten single zoospore lines were isolated from each transformant, and the *Avr4/6* amplicons were screened by restriction enzyme digestion and Sanger sequencing. T11, T18 and T32 yielded seven, 10 and 10 pure mutant lines, respectively (summarized in Table 1). All 27 pure lines showed a homogeneous sequence profile (Fig. S2, see Supporting Information), indicating that all were already homozygous, and carried the same mutation in both alleles. All 10 of the T32 lines were homozygous for the same mutation. The lines derived from T18 included nine lines homozygous for one *Avr4/6* mutation and one homozygous for a different mutation. The lines derived from T11 included two homozygous for one mutation (mut1) and five homozygous for a second mutation (mut2). The three remaining lines were heterozygous and biallelic, containing a third mutation paired with mut1 (Figs 3B and S2). No lines retained any wild-type alleles, either homozygous or heterozygous.

Each of the mutations consisted of a short indel, located specifically at the Cas9 cleavage site, i.e. between the 17th and 18th nucleotides of the sgRNA target site. Deletions of 1, 3 and 6 bp were observed, one contained a 1-bp insertion, and one combined a 3-bp deletion with a 1-bp replacement (Fig. 3B and Table 1).

We also tested the stability of the mutants by subculturing each of the single zoospore lines for at least three generations on medium without G418 selection. All of the mutated sites examined remained the same as in the first generation, based on the sequencing of the *Avr4/6* amplicons. Interestingly, one transformant, T47, which did not show obvious mutations in the first generation, acquired the same single adenine insertion as T32-3 after subculture of the unpurified transformant for one generation (Fig. S3, see Supporting Information), presumably because the sgRNA:Cas9 constructs were integrated into the genome and continued to actively cleave the target in each generation. Collectively, these results indicate that our CRISPR/Cas9 system can efficiently and specifically trigger the introduction of NHEJ mutations, typically short indels, into the *P. sojae* genome.

Homologous gene replacement stimulated by the CRISPR/Cas9 system

Donor DNA-mediated repair of sgRNA-guided Cas9 cuts has been proven to be an efficient method to facilitate gene replacements via HDR (Cong *et al*., 2013; Mali *et al*., 2013). To determine whether sgRNA:Cas9-mediated DSB could stimulate homologous recombination in *P. sojae*, we co-transformed the CRISPR constructs that were successfully used for mutation of *Avr4/6*, together with uncut donor DNA plasmids that contained the entire *NPT II* open reading frame (ORF) flanked by different lengths of the sequences surrounding the *Avr4/6* gene. An equimolar ratio of the three plasmids was used (Fig. 4A). As preliminary experiments had shown that expression of the *NPT II* gene from the *Avr4/6* promoter was insufficient for G418 selection, the *NPT II* gene was included in the Cas9 plasmid for the selection of transformants. We used homology arms consisting of three different lengths of 5' and 3' flanking sequences, namely 250 bp, 500 bp and 1 kb, to assess which would enable the highest recombination efficiency (Fig. 4B). The *NPT II* gene in the Cas9 expression plasmid served as a negative control, because it lacked any *Avr4/6* flanking sequences.

Following co-transformation and G418 enrichment, the bulk transformants were subjected to gDNA extraction and PCR analysis. PCR amplifications using primers located outside the *Avr4/6* homology arms and within the *NPT II* gene were used to detect homologous recombination events. The results suggested that HDR had occurred, but the frequency was variable depending on the length of the flanking sequences in the donor DNA plasmids. The transformant population generated with the 1-kb flanking sequences showed the highest frequency of gene replacement. The population generated with the 500-bp flanking sequences showed a much lower frequency compared with the 1-kb

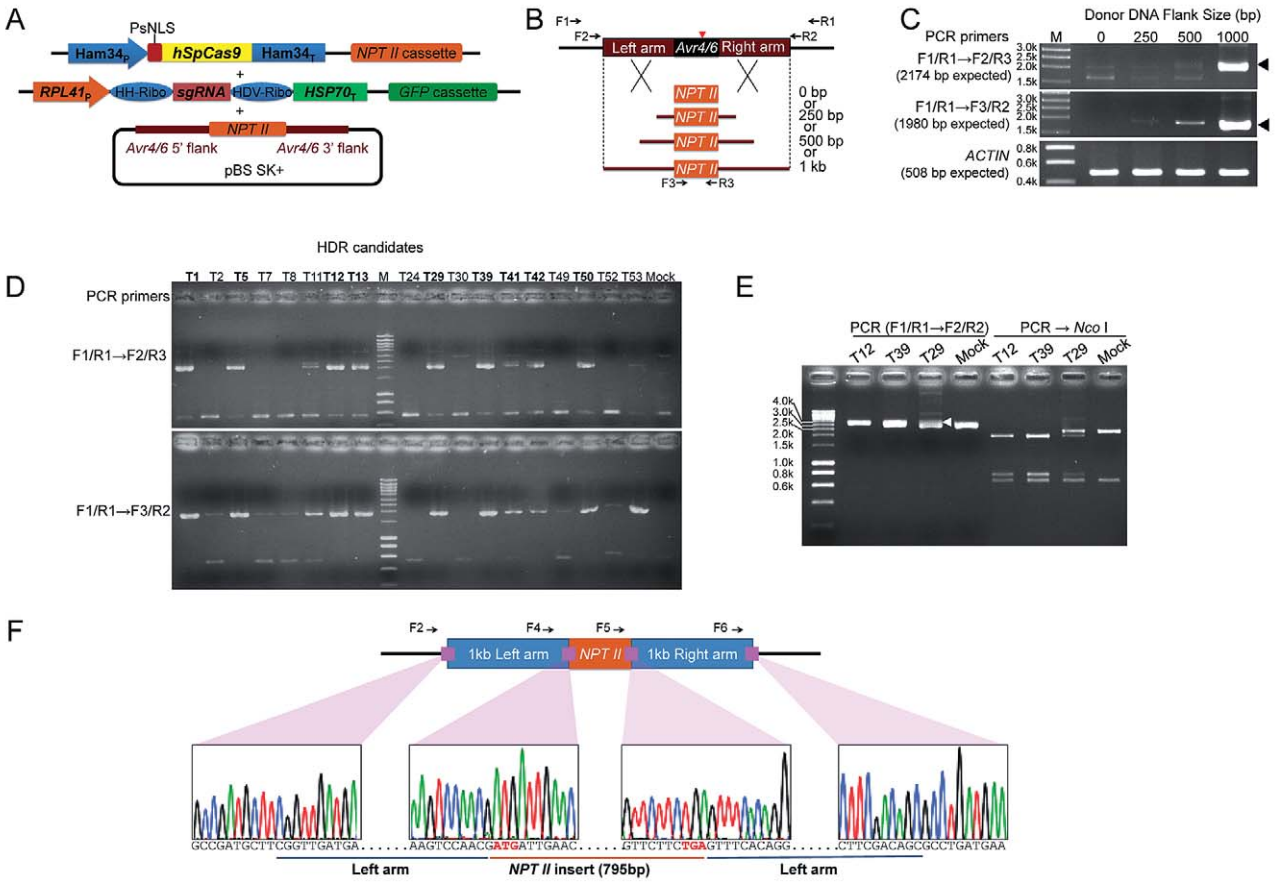


Fig. 4 Homology-directed repair (HDR)-mediated replacement of the *Avr4/6* open reading frame (ORF) with an *NPT II* ORF. (A) Strategy used for gene replacement. Plasmids containing a homologous donor DNA (*NPT II* with *Avr4/6* flanking sequences) were co-transformed with the Avr4/6 sgRNA-B_R and hSpCas9 constructs. (B) Three different sizes of the homology arms, 250 bp, 500 bp and 1 kb, flanking the *Avr4/6* locus, were used. The *NPT II* gene in the hSpCas9 expression plasmid served as the control (0-bp homologous arm, mock). Primers used to screen the HDR mutants and to validate the replaced region are shown as arrows. Primer pair F1/R1 and nested primer pairs F2/R3, F3/R2 and F2/R2 were used for HDR mutant screening. Red arrowhead indicates the CRISPR/Cas9 cleavage site (between 232 and 233 bp of *Avr4/6* ORF). (C) Analysis of genomic DNA from the pooled transformants produced using the four sizes of flanking sequences, employing nested PCR. Arrowheads indicate sizes expected if HDR has occurred. *ACTIN*, actin control for DNA quality. (D) Screening of individual HDR transformants generated with the 1-kb flanking sequence plasmid. The nine positive HDR mutants are highlighted in bold. (E) PCR analysis of representative zoospore-purified lines of HDR mutants, demonstrating that T12 and T39 are homozygotes, whereas T29 is a heterozygote. DNA sizes (bp) before restriction enzyme cleavage: WT, 2936; HDR mutant, 3359; after *Nco* I digestion: WT, 2273 + 663; HDR mutant, 1945 + 751 + 663. The arrowhead indicates the fragment amplified from the *NPT II*-replaced allele. Primer F4, Avr4/6_up500bp_PhusF (Table S1). (F) Sanger sequencing traces of junction regions confirming that the *Avr4/6* ORF was cleanly replaced by the *NPT II* ORF in a representative zoospore-purified clone (HDR-T12-1). Start and stop codons are indicated in bold red.

population. The population from the 250-bp flanking sequences showed very low recombination frequencies (Fig. 4C).

Next, to characterize HDR events in detail, we generated single *P. sojae* transformants derived from the 1-kb arm donor. After screening 68 individual G418 resistant transformants for GFP production, we identified 18 transformants bearing the two CRISPR components. Then, using nested primers specific for HDR events (Fig. 4B), we found evidence for gene replacement events in nine of the transformants (Fig. 4D). Sanger sequencing across the junctions of the flanking sequences and *NPT II* in the nested PCR products was also consistent with replacement of the *Avr4/6* ORF with the *NPT II* gene (data not shown). Three HDR mutants, namely HDR-T12, HDR-T29 and HDR-T39, that readily produced zoospores were selected for functional tests. After zoospore isolation, the *Avr4/6* region of each single zoospore line was examined by PCR amplification using primers flanking the two homologous arms and cleavage of the amplicon by the restriction enzyme *Nco* I. We found that all of the 11 single zoospore lines obtained from HDR-T12 and all eight obtained from HDR-T39 were homozygotes (Fig. 4E); this was further validated by Sanger sequencing (Fig. 4F). In contrast, all 20 of the single zoospore lines of T29 appeared to be heterozygotes that contained an HDR event in just one of the two *Avr4/6* alleles (Fig. 4E). This was further verified by PCR amplification using primers outside of the homology arms and in the

NPT II gene. More detailed analysis of three of the HDR-T29 lines revealed that the non-HDR alleles possessed the same mutation, an adenine deletion, in every case (Fig. S3), presumably caused by NHEJ.

Modified recognition of *Avr4/6* mutants by soybean carrying the *Rps*4 and *Rps*6 loci

In order to test the effects of the CRISPR/Cas9-induced *Avr4/6* mutations on *P. sojae* recognition by plants containing the *Rps*4 and *Rps*6 loci, the five homozygous NHEJ mutants and two homozygous HDR mutants were inoculated onto hypocotyls of soybean isolines containing *Rps*4 (L85-2352) or *Rps*6 (L89-1581) in a Williams background, as well as isolines containing *Rps*4 (HARO4272) or *Rps*6 (HARO6272) in a Harosoy background. At 4 days post-inoculation (dpi), the specific virulence of the different mutants was scored and analysed by Fisher's exact test (Table 2). We observed that the frame-shifted mutants T32-3 and T11-2-5 both showed increased killing of *Rps*4- or *Rps*6-containing soybean seedlings in both Williams and Harosoy backgrounds (Table 2). The increased killing of both *Rps*4 and *Rps*6 plants by T32-3 was statistically significant ($P < 0.01$), whereas the increased killing by T11-2-5 of *Rps*4, but not *Rps*6, plants was significant ($P < 0.05$). However, the increased killing in every case was still significantly ($P < 0.05$) less than the killing of *rps* plants lacking *Rps*4 or *Rps*6 (Table 2). Thus, T32-3 was scored as intermediate on *Rps*4 and *Rps*6 plants, whereas T11-2-5 was scored as avirulent and intermediate, respectively. The other NHEJ mutants, containing in-frame deletions, also showed increased killing of *Rps*4- and *Rps*6-containing plants, but significantly ($P < 0.03$) less killing than observed with *rps* plants. The *Avr4/6* mutant having a two-amino-acid deletion (T11-1) showed an intermediate phenotype that was close to fully virulent on *Rps*4 plants, whereas the two mutants with a single amino acid deletion (18-1 and 18-2) showed intermediate to avirulent phenotypes (Table 2).

The two homozygous HDR mutants (T12-1 and T39-1) both showed significantly ($P < 0.01$) more killing of *Rps*4- or *Rps*6-containing soybean seedlings in both Williams and Harosoy backgrounds (Table 2; Fig. 5), but the killing was significantly ($P < 0.05$) less than on *rps* plants. Thus, both mutants were scored as intermediate to virulent on *Rps*4 and *Rps*6.

DISCUSSION

Substantial numbers of oomycete genomes have now been sequenced, and even larger numbers are underway (Jiang and Tyler, 2012; Jiang *et al*., 2013; Kamoun *et al*., 2015). These genomes contain 15 000–25 000 genes, approximately one-half of which, in most species, show the rapid sequence divergence expected of infection-related genes (Baxter *et al*., 2010; Haas *et al*., 2009; Jiang and Tyler, 2012; Jiang *et al*., 2013; Tyler *et al*., 2006). To date, however, the tools available for the assessment of

Table 2 Characterization of the virulence of *Phytophthora sojae Avr4/6* non-homologous end-joining (NHEJ) and homology-directed repair (HDR) mutants on soybean.

Williams	Ws (*rps*)		L85-2352 (*Rps*4)				L89-1581 (*Rps*6)			
Strains	sv*	vir†	sv	*P* value 1‡	*P* value 2§	vir	sv	*P* value 1	*P* value 2	vir
WT	0/60	V	39/58	–	–	A	30/46	–	–	A
T11-1	0/40	V	7/47	0.014	<0.0001	I/V	12/35	<0.0001	0.0074	I/A
T11-2-5 fs¶	0/52	V	41/70	<0.0001	0.36	A	18/53	<0.0001	0.034	I/A
T18-1	1/40	V	18/53	0.0001	0.0006	I	15/32	<0.0001	0.16	A
T18-2	0/39	V	16/45	<0.0001	0.0016	I/A	12/26	<0.0001	0.21	A
T32-3 fs	0/54	V	6/60	0.029	<0.0001	I/V	18/51	<0.0001	0.0044	I/A
HDR-T12-1	0/20	V	9/28	0.0063	0.0027	I	8/32	0.0174	0.0006	I/V
HDR-T39-1	0/19	V	11/31	0.0035	0.0067	I	7/33	0.0390	0.0002	I/V
Harosoy	(1-7)1 (*rps*)		4272 (*Rps*4)				6272 (*Rps*6)			
Strains	sv	vir	sv	*P* value 1	*P* value 2	vir	sv	*P* value 1	*P* value 2	vir
WT	0/40	V	60/85	–	–	A	53/76	–	–	A
T11-1	0/38	V	5/37	0.025	<0.0001	I/V	15/39	<0.0001	0.0024	I/A
T11-2-5 fs	0/40	V	47/76	<0.0001	0.25	A	34/79	<0.0001	0.0011	I/A
T18-1	0/40	V	19/36	<0.0001	0.094	A	15/28	<0.0001	0.16	A
T18-2	0/39	V	20/35	<0.0001	0.20	A	16/40	<0.0001	0.0027	I/A
T32-3 fs	0/42	V	15/64	0.0004	<0.0001	I	25/64	<0.0002	0.0003	I
HDR-12-1	0/20	V	6/28	0.034	<0.0001	I/V	10/35	0.0090	<0.0001	I/V
HDR-39-1	0/21	V	10/33	0.0043	0.0001	I/V	9/31	0.0073	0.0002	I/V

*sv, soybean seedlings surviving after infection/total seedlings.

†vir, virulence of wild-type (WT) and mutants: V, virulent; A, avirulent; I, intermediate; I/A intermediate to avirulent; I/V, intermediate to virulent.

‡*P* value 1, difference between *Rps4*/*Rps*6 and the *rps* control.

§*P* value 2, difference between mutants and WT *P. sojae*.

¶fs, frame-shift mutant.

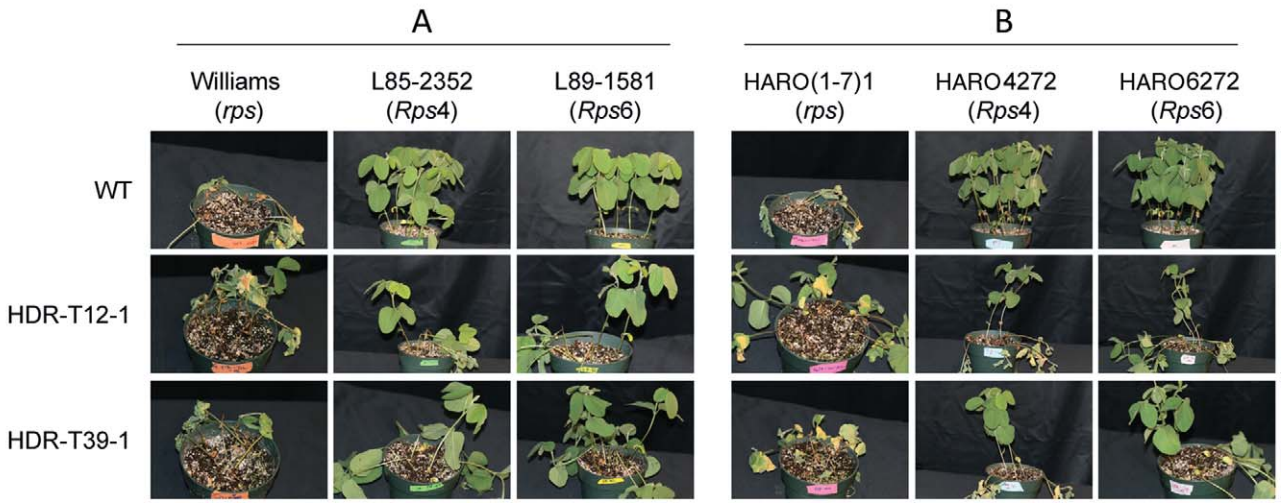


Fig. 5 Infection phenotypes of *Avr4/6* mutants. Representative photographs showing soybean seedlings inoculated on the hypocotyls with *Phytophthora sojae Avr4/6* homology-directed repair (HDR) mutants. L85-2352 and HARO4272 contain *Rps*4, whereas L89-1581 and HARO6272 contain *Rps*6. (A) Williams isolines. (B) Harosoy isolines. Photographs were taken at 4 days post-inoculation.

the functions of these genes have been limited to RNAi-mediated gene silencing and to the expression of ectopic transgenes.

Here, we have adapted the CRISPR/Cas9 sequence-specific nuclease technology for use in *P. sojae*. This involved overcoming several technical hurdles. One of these was the fact that commonly used mammalian NLSs do not function well in *P. sojae* and that *P. sojae* nuclear proteins use dispersed NLSs (Y. Fang and B. M. Tyler, unpublished data). The NLSs from several *P. sojae* nuclear proteins were delineated to the point that a small highly efficient NLS tag could be constructed (Y. Fang and B. M. Tyler, unpublished data). A second hurdle was that no RNA pol III promoters had been characterized in oomycetes, and that the U6 genes we attempted to use did not appear to be well transcribed under our transformation conditions. We solved this by using a strong RNA pol II promoter (*RPL41*) in conjunction with self-cleaving ribozymes to release the sgRNA from the RNA pol II transcript. A third hurdle was effective expression of the nuclease protein. Our initial attempts to use TALENs were blocked because the TALEN constructs were silenced extremely strongly in *P. sojae*, presumably because of their highly repetitive nature. The humanized SpCas9 protein was readily expressed, but, unexpectedly, the hSpCas9-GFP fusion protein used to validate expression was not effective in generating mutations in *P. sojae*, despite the fact that Cas9-GFP fusions have been used in other organisms, such as *Toxoplasma gondii* (Shen *et al*., 2014). We were successful only after we used the non-fused hSpCas9. A fourth hurdle was to identify effective sgRNAs. One of the two sgRNAs predicted using the *sgRNA Designer* web tool proved to be ineffective both *in vitro* and *in vivo*. This sgRNA, sgRNA-A, was found to be strongly self-complementary, potentially preventing hybridization with the target DNA. This observation, which is in agreement with the sgRNA design guideline proposed by Peng *et al*. (2015), underlines that sgRNAs with strong secondary structure predictions should be eliminated. We also observed a discrepancy between *in vitro* and *in vivo* assays of Cas9/sgRNA activity, namely that the sgRNA lacking ribozymes was functional in the *in vitro* assay, whereas only the sgRNA flanked by ribozymes was effective *in vivo*. There clearly is room for further optimization of sgRNA design.

Using a single sgRNA, we were able to generate small indels at the site of the Cas9 cleavage. Although these were useful, future attempts to disrupt genes with large deletions will probably require a pair of sgRNAs, or the use of HDR to introduce specific mutations or to replace the gene entirely, as we did when replacing *Avr4/6* with *NPT II*. Of interest is the fact that, from two of our transformant lines, we were able to recover two or three different mutations. Several of these mutations were segregated into different single zoospore lines, indicating that the mutations had occurred in different nuclei of the regenerating protoplasts. One set of lines was biallelic, indicating that two different mutations had occurred in the same nucleus. Also of interest is the observation that most of the mutations were recovered as homozygotes in this diploid organism. We speculate that, once a mutation occurred in one allele that made it resistant to further Cas9 cleavage, cleavage of the remaining unmodified allele by Cas9 in most cases led to gene conversion of that allele to match the first allele. During subculture and infection assays of all of our Cas9-expressing mutants, we did not observe negative effects on *P. sojae* growth or overall virulence. Future work, including meiotic analysis, careful examination of possible off-target effects and targeting of additional genes, will expand our understanding of the utility and any limitations of the technology in *P. sojae* and other oomycetes.

CRISPR-mediated gene disruptions and gene replacements will find numerous applications in *P. sojae* and other oomycetes. With careful design of sgRNAs, single members of closely related gene families could be eliminated, or tags for transcriptional measurements could be introduced, to determine their individual contributions. Alternatively, entire gene clusters could be eliminated with a pair of sgRNAs. Gene replacements will enable mutations of all kinds, including promoter mutations and epitope and fluorescent protein tags, to be introduced into the endogenous gene, where expression and phenotypes will not be confounded by position effects, overexpression artefacts or contributions from the unmodified native gene. Targeted gene insertions are also expected to solve a long-standing problem in some oomycetes, such as *P. sojae*, in which ectopic transgenes are invariably poorly expressed, even from the strongest promoter. Gene disruptions will also be useful for the creation of a much wider choice of selectable markers for transformation through the creation of auxotrophic mutants. Gene deletions could also be used to remove integrated transgenes, so that selectable markers, such as *NPT II*, can be recycled for repeated transformation experiments.

Some of the advantages of these CRISPR-enabled approaches are illustrated by insights gained from our manipulation of the *Avr4/6* gene. The elimination of the gene by replacement with *NPT II* confirms that *Avr4/6* makes a major contribution to recognition of the pathogen by plants containing the *Rps*4 and *Rps*6 loci, consistent with the findings of Dou *et al*. (2010). However, the mutants did not kill the *Rps*4- and *Rps*6-containing plants as completely as they killed *rps* plants lacking *Rps*4 and *Rps*6. The *Rps*4- and *Rps*6-containing isolines were produced by introgression, not by transformation with individual *R* genes (Sandhu *et al*., 2004). As the *Rps*4 and *Rps*6 loci, which are allelic, both contain many nucleotide-binding and leucine-rich repeat (NB-LRR) genes (Sandhu *et al*., 2004), we speculate that additional NB-LRR genes at these loci (or even the *Rps*4 and *Rps*6 genes themselves) can recognize additional effectors produced by the *P. sojae* strain used in these studies (P6497). Dou *et al*. (2010) also observed that *P. sojae* strains silenced for Avr4/6 did not completely kill *Rps*4- and *Rps*6-containing lines, but these observations were ascribed to incomplete silencing of *Avr4/6*. With the availability of complete *Avr4/6* deletion mutants, we can now more confidently conclude that the *Rps*4 and *Rps*6 loci make additional contributions to resistance other than through the recognition of Avr4/6.

The two frame-shift mutants killed *Rps*6 plants nearly as well as did the HDR mutants (62% combined killing versus 74%), indicating that these mutants were no longer recognized by *Rps*6-containing plants. T32-3 killed *Rps*4 plants more effectively than did the two HDR mutants (83% versus 74% killing), but T11-2-5 was clearly avirulent on *Rps*4 plants (40% versus 74%; wild-type, 31%). The explanation for this difference may lie in the observation by Dou *et al*. (2010) that the N-terminal domain of Avr4/6, up to and including the dEER motif, is sufficient for recognition by *Rps*4 plants, whereas recognition by *Rps*6 plants requires the C-terminus. As the site of the CRISPR-induced NHEJ mutations is immediately upstream of the dEER motif, the +1 frame-shift in T32-3 retained the N-terminal domain but eliminated the dEER motif, presumably abolishing effector entry into the plant (Dou *et al*., 2008b). The –1 frame-shift in T11-2-5 also eliminated the dEER motif, but in its place created a highly positively charged sequence (RKKRNARSR). Thus, we speculate that this positively charged sequence may act as a surrogate cell entry sequence (Dou *et al*., 2008b; Kale *et al*., 2010; Milletti, 2012; Snyder and Dowdy, 2004) delivering the N-terminal fragment for recognition by *Rps*4.

Of the three in-frame deletions, the two single-amino-acid deletion mutants scored as intermediate to avirulent on both *Rps*4 and *Rps*6 plants, suggesting that recognition was only slightly impaired. The two-amino-acid deletion mutant (T11-1) showed a stronger loss of Avr4/6 function, possibly as a result of disruption of the structure of Avr4/6.

In summary, the adaptation of CRISPR/Cas9-mediated gene targeting to oomycetes is expected to rapidly advance the functional analysis of these extremely destructive and important plant and animal pathogens.

EXPERIMENTAL PROCEDURES

Phytophthora sojae strains and growth conditions

The reference *P. sojae* isolate P6497 (Race 2) used in this study was routinely grown and maintained in cleared V8 medium at 25 °C in the dark. Zoospores were induced and isolated from approximately 1-week-old cultures grown on clarified V8 agar, as described previously (Judelson *et al*., 1993a). Single *P. sojae* transformants were incubated in 12-well plates containing V8 medium supplemented with 50 μg/mL G418 (Geneticin, AG Scientific, San Diego, California, USA) for 2–3 days before small-scale genomic DNA extraction.

sgRNA design

sgRNA target sites were selected according to the web tool *sgRNA Designer* (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design; Doench *et al*., 2014). Potential off-target sites were checked using the FungiDB (www.fungidb.org) alignment search tool (BLASTN) against the *P. sojae* genome and visual inspection of the results. Sequences that perfectly matched the final 12 nucleotides of the target sequence and NGG PAM sequence were discarded (Cong *et al*., 2013). Ribozymes were designed according to Gao and Zhao (2014). The first six nucleotides of the HH ribozyme were designed to be the reverse complement of the first six nucleotides of the sgRNA target sequences.

Plasmid construction

All the primers used in this study are listed in Table S1 and further details on plasmid construction are described in Methods S1 (see Supporting

Information). A map and sequence file for the plasmid backbones used for the expression of Cas9 and the sgRNAs targeting the *Avr4/6* locus can be found in Fig. S4 and the supplemental sequences in Appendix S1.

sgRNA:Cas9 *in vitro* activity assay

To test the activity of the designed sgRNAs, an *in vitro* cleavage assay was carried out (Gao and Zhao, 2014). Briefly, sgRNA was *in vitro* transcribed through run-off reactions with T7 RNA polymerase using the MEGAshortscript™ T7 kit (Ambion, Austin, TX, USA) according to the manufacturer's manual. Templates for sgRNA synthesis were generated by PCR amplification from the sgRNA expression plasmid pYF2.2-GFP-sgRNAs (Table S1). The target DNA was amplified from pCR2.1-Avr4/6 using primers M13F and M13R (Appendix S1). SpCas9 nuclease was purchased from New England Biolabs Inc., Ipswich, MA, USA, and the cleavage assay was performed according to the product manual.

Improved transformation of *P. sojae*

Polyethylene glycol (PEG)-mediated protoplast transformations were conducted using a modification of the previously described methods (Dou *et al*., 2008a; Mcleod *et al*., 2008). Two- to four-day-old *P. sojae* mycelial mats, cultured in nutrient pea broth, were harvested and pretreated with 0.8 M mannitol for 10 min, and then digested in 20 mL of enzyme solution [0.4 M mannitol, 20 mM KCl, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.7, 10 mM $CaCl_2$, 0.5% Lysing Enzymes from *Trichoderma harzianum* (Sigma L1412: St Louis, MO, USA) and 0.5% CELLULYSIN® Cellulase (Calbiochem 219466: San Diego, California, USA)] for approximately 40 min at room temperature with gentle shaking. The mixture was filtered through a Falcon™ Nylon Mesh Cell Strainer (BD Biosciences: San Diego, CA, USA) and protoplasts were pelleted by centrifugation at 1200 ***g*** for 2 min in a Beckman Coulter, Miami, FL, USA benchtop centrifuge with swing buckets. After washing with 30 mL of W5 solution (5 mM KCl, 125 mM $CaCl_2$, 154 mM NaCl and 177 mM glucose), protoplasts were resuspended in 10 mL of W5 solution and left on ice for 30 min. Protoplasts were collected by centrifugation at 1200 ***g*** for 2 min in the Beckman centrifuge and resuspended at 10^6/mL in MMg solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES, pH 5.7). DNA transformation was conducted in a 50 mL Falcon tube, where 1 mL protoplasts were well mixed with 20–30 μg of DNA for single plasmid transformation. For co-transformation experiments, 20–30 μg of the plasmid carrying the *NPT II* selectable marker gene were used, together with an equimolar ratio of any other DNAs included. Then, three successive aliquots of 580 μL each of freshly made PEG solution (40% PEG 4000 v/v, 0.2 M mannitol and 0.1 M $CaCl_2$) were slowly pipetted into the protoplast suspension and gently mixed. After 20 min incubation on ice, 10 mL of pea broth containing 0.5 M mannitol were added, and the protoplasts were regenerated overnight at 18 °C in the dark. For the production of stable transformants, the regenerated protoplasts were collected by centrifugation at 2000 ***g*** for 2 min in the Beckman centrifuge, and then resuspended and evenly divided into three Falcon tubes containing 50 mL of liquid pea broth containing 1% agar (42 °C), 0.5 M mannitol and 50 μg/mL G418 (AG Scientific, San Diego, CA, USA). The resuspended protoplasts were then poured into empty 60 mm × 15 mm Petri dishes. Mycelial colonies could be observed after 2 days of incubation at 25 °C in the dark. The visible transformants were transferred to V8 liquid medium containing 50 μg/mL G418 and propagated for 2–3 days at 25 °C prior to analysis. For transient expression, 50 μg/mL G418 was usually added to the regeneration medium after overnight recovery to enrich the positive transformants. After 1 day of incubation at 25 °C in the dark, hyphae were collected for gDNA extraction.

Detection and quantification of targeted mutagenesis

To detect the results of targeted mutagenesis in transformants, total gDNA was extracted from pooled or individual *P. sojae* transformants. For pooled transformants, 48 h after transformation, 1 mL of the mycelial culture was pelleted, resuspended in 500 mL of lysis buffer [200 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 200 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 2% sodium dodecylsulfate (SDS), plus 0.1 mg/mL RNaseA added prior to use] and broken by vortexing with 0.5-mm glass beads. For individual transformants, an approximately 7-mm-diameter clump of *P. sojae* hyphae was blotted dry on Kimwipe™ paper, frozen in liquid nitrogen, ground to a powder using a polypropylene pestle, and then resuspended in 500 μL of lysis buffer. Hyphal lysates were incubated at 37 °C for 30 min for RNA digestion, and the DNA was recovered by phenol–chloroform extraction and isopropanol precipitation.

All PCR amplifications were conducted using Phusion® high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) in order to exclude the possibility of mutations caused during PCR amplification. Generally, approximately 10 ng of gDNA was used as PCR template. Nested PCR was conducted if necessary, using 1 : 1000 diluted PCR products as a DNA template for the second round.

To detect NHEJ mutations, the entire 372-bp *Avr4/6* ORF was amplified and examined by digestion with the relevant restriction enzyme. For pooled transformants, a nested PCR was performed to enrich the mutated target before sequencing; this step was not needed for individual transformants, including single zoospore lines. To detect HDR events in pooled and individual transformants (other than zoospore lines), primers located outside the *Avr4/6* homology arms and in the *NPT II* gene were used. For screening single zoospore lines, PCR was performed by using primers only outside the homology arms. In both cases, nested PCR was carried out for efficient amplification of the targets. PCR products were sequenced directly by the Sanger dideoxy method in the Oregon State University Center for Genome Research and Biocomputing, Corvallis, OR, USA.

Confocal microscopy

Laser scanning confocal microscopy (Carl Zeiss, Oberkochen, Germany, LSM 780 NLO) was used to examine the expression and subcellular localization of hSPCas9 fused to the NLS and to GFP. Living hyphae were picked from liquid cultures after 2–3 days of growth of transformants. Samples were stained with 4',6-diamidino-2-phenylindole (DAPI) for 20 min in the dark (Talbot, 2001) before microscopy examination. Images were captured using a 63× oil objective with excitation/emission settings of 405 nm/410–490 nm for DAPI and 488 nm/510–535 nm for GFP.

Infection assays

The ability of *Avr4/6* mutants to infect soybean plants carrying *Rps*4 and *Rps*6 was evaluated by hypocotyl inoculation, as described previously (Dou *et al*., 2010). The wild-type and mutant *P. sojae* strains were grown on V8 plates without G418 selection for approximately 5 days. Soybean cultivars HARO(1-7)1 (*rps*), HARO4272 (Harosoy background, *Rps*4, *Rps*7), HARO6272 (Harosoy background, *Rps*6, *Rps*7), Williams (*rps*), L85-2352 (Williams background, *Rps*4) and L89-1581 (Williams background, *Rps*6) were used. Each pathogenicity test was performed in triplicate, each replicate consisting of at least 19 seedlings. A strain was considered to be avirulent if the number of inoculated *Rps*4 or *Rps*6 seedlings surviving was significantly higher than the number of surviving seedlings lacking the *Rps* gene, as determined by Fisher's exact test (Sokal and Rohlf, 1995), and the number was not significantly different from the number of surviving seedlings inoculated with the unmodified control strain P6497. A strain was considered to be virulent if the number of surviving *Rps*4 or *Rps*6 seedlings was not significantly different from the number of surviving *rps* seedlings, and was significantly fewer than the number of seedlings surviving P6497 inoculation. A strain was considered to be intermediate if the number of surviving *Rps*4 or *Rps*6 seedlings was not significantly different from the number of surviving *rps* seedlings, and also was not significantly different from the number of seedlings surviving P6497 inoculation. A strain was also considered to be intermediate if the number of surviving *Rps*4 or *Rps*6 seedlings was significantly greater than the number of surviving *rps* seedlings, and also significantly fewer than the number of seedlings surviving P6497 inoculation. Intermediate phenotypes were further designated intermediate/virulent or intermediate/avirulent if the *P* values indicating a difference from virulent or avirulent controls differed by more than 10-fold.

ACKNOWLEDGEMENTS

We thank F. Arredondo, S. Taylor and D. Wellappili (Oregon State University, Corvallis, OR, USA) for experimental assistance, M. A. Saghai-Maroof (Virginia Tech, Blacksburg, VA, USA) for soybean seed and H. Judelson (University of California-Riverside, CA, USA) and members of the Tyler Laboratory for useful advice. We acknowledge the Sequencing and Confocal Microscopy Facilities of the Center for Genome Research and Biocomputing at Oregon State University. This work was supported in part by grant 2011-68004-30104 from the Agriculture and Food Research Initiative of the US Department of Agriculture (USDA) National Institute for Food and Agriculture.

REFERENCES

Ah-Fong, A.M., Bormann-Chung, C.A. and Judelson, H.S. (2008) Optimization of transgene-mediated silencing in *Phytophthora infestans* and its association with small-interfering RNAs. *Fungal Genet. Biol.* **45**, 1197–1205.

Baxter, L., Tripathy, S., Ishaque, N., Boot, N., Cabral, A., Kemen, E., Thines, M., Ah-Fong, A., Anderson, R., Badejoko, W., Bittner-Eddy, P., Boore, J.L., Chibucos, M.C., Coates, M., Dehal, P., Delehaunty, K., Dong, S., Downton, P., Dumas, B., Fabro, G., Fronick, C., Fuerstenberg, S.I., Fulton, L., Gaulin, E., Govers, F., Hughes, L., Humphray, S., Jiang, R.H.Y., Judelson, H., Kamoun, S., Kyung, K., Meijer, H., Minx, P., Morris, P., Nelson, J., Phuntumart, V., Qutob, D., Rehmany, A., Rougon-Cardoso, A., Ryden, P., Torto-Alalibo, T., Studholme, D., Wang, Y., Win, J., Wood, J., Clifton, S.W., Rogers, J., Van den Ackerveken, G., Jones, J.D.G., McDowell, J.M., Beynon, J. and Tyler, B.M. (2010) Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science*, **330**, 1549–1551.

Brinkman, E.K., Chen, T., Amendola, M. and van Steensel, B. (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* **42**, e168.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.

Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, I., Sullender, M., Ebert, B.L., Xavier, R.J. and Root, D.E. (2014) Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol.* **32**, 1262–1267.

Dou, D., Kale, S.D., Wang, X., Chen, Y., Wang, Q., Wang, X., Jiang, R.H., Arredondo, F.D., Anderson, R.G., Thakur, P.B., McDowell, J.M., Wang, Y. and Tyler, B.M. (2008a) Conserved C-terminal motifs required for avirulence and suppression of cell death by *Phytophthora sojae* effector Avr1b. *Plant Cell*, **20**, 1118–1133.

Dou, D., Kale, S.D., Wang, X., Jiang, R.H.Y., Bruce, N.A., Arredondo, F.D., Zhang, X. and Tyler, B.M. (2008b) RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell*, **20**, 1930–1947.

Dou, D., Kale, S.D., Liu, T., Tang, Q., Wang, X., Arredondo, F.D., Basnayake, S., Whisson, S., Drenth, A., Maclean, D. and Tyler, B.M. (2010) Different domains of *Phytophthora sojae* effector Avr4/6 are recognized by soybean resistance genes Rps 4 and Rps 6. *Mol. Plant–Microbe Interact.* **23**, 425–435.

Erwin, D.C. and Ribeiro, O.K. (1996) *Phytophthora Diseases Worldwide*. St. Paul, MN: American Phytopathological Society.

Fang, Y. and Tyler, B.M. (2015) The Oomycete *Phytophthora sojae* uses non-canonical nuclear localization signals to direct proteins into the nucleus. *Fung. Genet. Rep.* **61** (Suppl.), Abstract #165.

Gaj, T., Gersbach, C.A. and Barbas, C.F., 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* **31**, 397–405.

Gao, Y. and Zhao, Y. (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. *J. Integr. Plant Biol.* **56**, 343–349.

Gao, Y., Zhang, Y., Zhang, D., Dai, X., Estelle, M. and Zhao, Y. (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or Arabidopsis development. *Proc. Natl. Acad. Sci. USA*, **112**, 2275–2280.

Gijzen, M., Förster, H., Coffey, M.D. and Tyler, B. (1996) Cosegregation of Avr4 and Avr6 in *Phytophthora sojae*. *Can. J. Bot.* **74**, 800–802.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H.Y., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T.O., Ah-Fong, A.M.V., Alvarado, L., Anderson, V.L., Armstrong, M.R., Avrova, A.O., Baxter, L., Beynon, J.L., Boevink, P.C., Bollmann, S.R., Bos, J.I.B., Bulone, V., Cai, G., Cakir, C., Carrington, J.C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M.I.A., Fugelstad, J., Gilroy, E.M., Gnerre, S., Green, P.J., Grenville-Briggs, L.J., Griffith, J.M., Grünwald, N.J., Horn, K., Horner, N.R., Hu, C.H., Huitema, E., Jeong, D.H., Jones, A.M.E., Jones, J.D.G., Jones, R.W., Karlsson, E.K., Kunjeti, S.G., Lamour, K., Liu, Z., Ma, L.J., Maclean, D.J., Chibucos, M.C., McDonald, H., McWalters, J., Meijer, H.J.G., Morgan, W., Morris, P.F., Munro, C.A., O'Neill, K., Ospina-Giraldo, M.D., Pinzon, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D.C., Schumann, U.D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D.J., Sykes, S., Thines, M., van de Vondervoort, P.J.I., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W.E., Meyers, B.C., van West, P., Ristaino, J.B., Govers, F., Birch, P.R.J., Whisson, S.C., Judelson, H.S. and Nusbaum, C. (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature*, **461**, 393–398.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson R.T., Yeh, J.R. and Joung, J.K. (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227–229.

Jacobs, J.Z., Ciccaglione, K.M., Tournier, V. and Zaratiegui, M. (2014) Implementation of the CRISPR-Cas9 system in fission yeast. *Nat. Commun.* **5**, 5344.

Jiang, R.H. and Tyler, B.M. (2012) Mechanisms and evolution of virulence in oomycetes. *Annu. Rev. Phytopathol.* **50**, 295–318.

Jiang, R.H., de Bruijn, I., Haas, B.J., Belmonte, R., Löbach, L., Christie, J., van den Ackerveken, G., Bottin, A., Dumas, B., Fan, L., Gaulin, E., Govers, F.,

Grenville-Briggs, L.J., **Horner, N.R.**, **Levin, J.Z.**, **Mammella, M.**, **Meijer, H.J.G.**, **Morris, P.**, **Nusbaum, C.**, **Oome, S.**, **Rooyen, D.V.**, **Saraiva, M.**, **Secombes, C.J.**, **Seidl, M.F.**, **Snel, B.**, **Stassen, J.**, **Sykes, S.**, **Tripathy, S.**, **van den Berg, H.**, **Vega-Arreguin, J.C.**, **Wawra, S.**, **Young, S.**, **Zeng, Q.**, **Dieguez-Uribeondo, J.**, **Russ, C.**, **Tyler, B.M.** and **van West, P.** (2013) Distinctive expansion of potential virulence genes in the genome of the oomycete fish pathogen *Saprolegnia parasitica*. *PLoS Genet*. **9**, e1003272.

Judelson, H.S. (1997) The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. *Fungal Genet. Biol.* **22**, 65–76.

Judelson, H.S., Coffey, M.D., Arredondo, F.R. and Tyler, B.M. (1993a) Transformation of the oomycete pathogen *Phytophthora-megasperma* F-Sp *glycinea* occurs by DNA integration into single or multiple chromosomes. *Curr. Genet.* **23**, 211–218.

Judelson, H.S., Dudler, R., Pieterse, C.J., Unkles, S.E. and Michelmore, R.W. (1993b) Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects. *Gene*, **133**, 63–69.

Kale, S.D., Gu, B., Capelluto, D.G.S., Dou, D.-L., Feldman, E., Rumore, A., Arredondo, F.D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C.B., Shan, W. and Tyler, B.M. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell*, **142**, 284–295.

Kamoun, S., Furzer, O., Jones, J.D.G., Judelson, H.S., Ali, G.S., Dalio, R.J.D., Roy, S.G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.-R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B.M., Grünwald, N.J., Mukhtar, M.S., Tomé, D.F.A., Tör, M., Van den Ackerveken, G., McDowell, J., Daayf, F., Fry, W.E., Lindqvist-Kreuze, H., Meijer, H.J.G., Petre, B., Ristaino, J., Yoshida, K., Birch, P.R.J. and Govers, F. (2015) The Top 10 oomycete pathogens in molecular plant pathology. *Mol. Plant Pathol.* **16**, 413–434.

Lamour, K.H., Finley, L., Hurtado-Gonzales, O., Gobena, D., Tierney, M. and Meijer, H.J. (2006) Targeted gene mutation in *Phytophthora* spp. *Mol. Plant–Microbe Interact.* **19**, 1359–1367.

Liu, R., Chen, L., Jiang, Y., Zhou, Z. and Zou, G. (2015) Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. *Cell Discov.* **1**, 15 007.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E. *et al*. (2013) RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826.

Mcleod, A., Fry, B.A., Zuluaga, A.P., Myers, K.L. and Fry, W.E. (2008) Toward improvements of oomycete transformation protocols. *J. Eukaryot. Microbiol.* **55**, 103–109.

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., Dulay, G.P., Hua, K.L., Ankoudinova, I., Cost, G.J., Urnov, F.D., Zhang, H.S., Holmes, M.C., Zhang, L., Gregory, P.D. and Rebar, E.J. (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* **29**, 143–148.

Milletti, F. (2012) Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov. Today*, **17**, 850–860.

Peng, D., Kurup, S.P., Yao, P.Y., Minning, T.A. and Tarleton, R.L. (2015) CRISPR-Cas9-mediated single-gene and gene family disruption in *Trypanosoma cruzi*. *MBio*, **6**, e2097–e2014.

Sandhu, D., Gao, H., Cianzio, S. and Bhattacharyya, M.K. (2004) Deletion of a disease resistance nucleotide-binding-site leucine-rich-repeat-like sequence is associated with the loss of the *Phytophthora* resistance gene Rps4 in soybean. *Genetics*, **168**, 2157–2167.

Shen, B., Brown, K.M., Lee, T.D. and Sibley, L.D. (2014) Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. *MBio*, **5**, e01114.

Snyder, E.L. and Dowdy, S.F. (2004) Cell penetrating peptides in drug delivery. *Pharm. Res.* **21**, 389–393.

Sokal, R.R. and Rohlf, F.J. (1995) *Biometry: The Principles and Practice of Statistics in Biological Research*, San Francisco, CA: WH. Freeman & Co.

Talbot, N.J. (2001) *Molecular and Cellular Biology of Filamentous Fungi: A Practical Approach*, New York: Oxford University Press.

Tyler, B.M. (2001) Genetics and genomics of the oomycete–host interface. *Trends Genet.* **17**, 611–614.

Tyler, B.M. (2007) *Phytophthora sojae*: root rot pathogen of soybean and model oomycete. *Mol. Plant Pathol.* **8**, 1–8.

Tyler, B.M. and Gijzen, M. (2014) The *Phytophthora sojae* genome sequence: foundation for a revolution. In: *Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens* (Dean, R.A., Lichens-Park, A. and Kole, C., eds), pp. 133–157, New York: Springer.

Tyler, B.M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R.H.Y., Aerts, A., Arredondo, F.D., Baxter, L., Bensasson, D., Beynon, J.L., Chapman, J., Damasceno, C.M.B., Dorrance, A.E., Dou, D., Dickerman, A.W., Dubchak, I.L., Garbelotto, M., Gijzen, M., Gordon, S.G., Govers, F., Grünwald, N.J., Huang, W., Ivors, K.L., Jones, R.W., Kamoun, S., Krampis, K., Lamour, K.H., Lee, M.-K., McDonald, W.H., Medina, M., Meijer, H.J.G., Nordberg, E.K., Maclean, D.J., Ospina-Giraldo, M.D., Morris, P.F., Phuntumart, V., Putnam, N.H., Rash, S., Rose, J.K.C., Sakihama, Y., Salamov, A.A., Savidor, A., Scheuring, C.F., Smith, B.M., Sobral, B.W.S., Terry, A., Torto-Alalibo, T.A., Win, J., Xu, Z., Zhang, H., Grigoriev, I.V., Rokhsar, D.S. and Boore, J.L. (2006) *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science*, **313**, 1261–1266.

Upadhyay, S.K., Kumar, J., Alok, A. and Tuli, R. (2013) RNA-guided genome editing for target gene mutations in wheat. *G3 (Bethesda)* **3**, 2233–2238.

Vyas, V.K., Barrasa, M.I. and Fink, G.R. (2015) A *Candida albicans* CRISPR system permits genetic engineering of essential genes and gene families. *Sci. Adv.* **1**, e1500248.

Wagner, J.C., Platt, R.J., Goldfless, S.J., Zhang, F. and Niles, J.C. (2014) Efficient CRISPR-Cas9-mediated genome editing in *Plasmodium falciparum*. *Nat. Methods*, **11**, 915–918.

Wang, Q., Han, C., Ferreira, A.O., Yu, X., Ye, W., Tripathy, S., Kale, S.D., Gu, B., Sheng, Y., Sui, Y., Wang, X., Zhang, Z., Cheng, B., Dong, S., Shan, W., Zheng, X., Dou, D., Tyler, B.M. and Wang, Y. (2011) Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell*, **23**, 2064–2086.

Whisson, S., Drenth, A., Maclean, D. and Irwin, J. (1994) Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. *Curr. Genet.* **27**, 77–82.

Whisson, S.C., Avrova, A.O., Van West, P. and Jones, J.T. (2005) A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. *Mol. Plant Pathol.* **6**, 153–163.

Zhang, C., Xiao, B., Jiang, Y., Zhao, Y., Li, Z., Gao, H., Ling, Y., Wei, J., Li, S., Lu, M., Su, X., Cui, H. and Yuan, J. (2014) Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. *MBio*, **5**, e01414.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 *Phytophthora* U6 promoter evaluation. (A) Alignment of selected oomycete U6 genes, showing that U6 transcripts are highly conserved. The numbers of U6 genes were variable in different oomycete species: eight in *P. sojae P6497* (*PsojP6497*), 127 in *P. infestans* (*PinfT30-4*), five in *P. capsici LT1534* (*PcapLT1534*), one in *P. parasitica* (Ppar INRA-310) and one in *Hyaloperonospora arabidopsidis* (*HaraEmoy2*). Genome data were obtained from the fungidb.org website. (B) Alignment of the eight annotated *P. sojae* U6 genes. PsU6-1 was used to test promoter activity. The red lines indicate the border of the upstream and downstream tRNAs. (C) One of the 127 *P. infestans* U6 genes cloned to test U6 promoter activity. Top: position of the PiU6 gene on *P. infestans* T30-4 Supercontig 65. Bottom: PiU6 sequence used for promoter activity test. The putative U6 coding region is underlined; a putative TATA-box is indicated in red. (D) The plasmid used for testing the functions of the PsU6-1 and PiU6 promoters in *P. sojae*. Residues 1–150 bp of *eGFP* (*enhanced green fluorescent protein*) were used as a transcription detection marker. Arrows indicate the primer pair U6GFP_F and U6GFP_R used for cloning the *eGFP* fragment and also for the detection of U6 transcripts by reverse transcription-polymerase chain reaction (RT-PCR). The *Eco*NI

restriction enzyme site used for insertion of the GFP detection marker is underlined in (A) and (B) and double underlined in (C).

Fig. S2 Representative sequencing chromatograms of the *Avr4/6* mutations in the single zoospore purified mutants. Regions in the box show the single guide RNA (sgRNA) target sites within the *Avr4/6* gene. The unambiguous sequencing profiles indicate that these mutant lines are all homozygous.

Fig. S3 Sanger sequencing profiles revealing that the subcultured Cas9:sgRNA transformant T47 (NHEJ-T47) and homology-directed repair (HDR) mutant T29 (HDR-T29-2) had non-homologous end-joining (NHEJ) mutations (1-bp insertion and deletion, respectively). Red triangles indicate the differences between wild-type (WT) and mutants.

Fig. S4 Plasmid backbones used for the expression of *hSpCas9* and single guide RNA (sgRNA) in *Phytophthora sojae*. (A) pYF2-2XGFP is used for tracking the subcellular localization and expression of PsNLS-fused *hSpCas9*. PsNLS is inserted into *Sac*II and *Spe*I sites. *hSpCas9* is inserted into *Spe*I and *Afl*II sites for subcellular localization examination, and *Spe*I and *Apa*I sites for CRISPR expression. (B) pYF2.2-GFP is used for the expression of sgRNA (inserted into *Nhe*I and *Age*I sites).

Table S1 Sequences of the oligonucleotides used in this study.

Appendix S1 Supplemental sequences. Sequences of plasmids used in this study.

Methods S1 Generation of *Phytophthora sojae* CRISPR/Cas9 plasmids.