Defining transgene insertion sites and off-target effects of homology-based gene silencing informs the application of functional genomics tools in *Phytophthora infestans*

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ABSTRACT

DNA transformation and homology-based transcriptional silencing are frequently used to assess gene function in *Phytophthora.* Since unplanned side-effects of these tools are not well-characterized, we used *P. infestans* to study plasmid integration sites and whether knockdowns caused by homology-dependent silencing spreads to other genes. Insertions occurred both in gene-dense and gene-sparse regions but disproportionately near the 5' ends of genes, which disrupted native coding sequences. Microhomology at the recombination site between plasmid and chromosome was common. Studies of transformants silenced for twelve different gene targets indicated that neighbors within 500-nt were often co-silenced, regardless of whether hairpin or sense constructs were employed and the direction of transcription of the target. However, *cis*-spreading of silencing did not occur in all transformants obtained with the same plasmid. Genome-wide studies indicated that unlinked genes with partial complementarity with the silencing-inducing transgene were not usually down-regulated. We learned that hairpin or sense transgenes were not co-silenced with the target in all transformants, which informs how screens for silencing should be performed. We conclude that transformation and gene silencing can be reliable tools for functional genomics in *Phytophthora* but must be used carefully, especially by testing for the spread of silencing to genes flanking the target.

INTRODUCTION

Strategies for deciphering the roles of genes in all organisms including plant pathogens and their hosts involve tools for functional genomics. These approaches commonly employ gene transfer methods to knock-down, knock-out, or overexpress the gene of interest (Jiang et al. 2013). Contributing to the success of these tactics is an understanding of the nature of DNA transformation, including its unplanned consequences. Studies across multiple kingdoms have shown that transformation may cause chromosomal rearrangements, gene silencing methods (including RNAi) may affect off-targets, gene editing may modify unintended loci, and regenerants may exhibit somaclonal variation (Doench et al. 2016; Firon et al. 2002; Kaelin 2012; Neelakandan and Wang 2012). An awareness of these complications helps direct the judicious use of functional genomics tools.

Methods for DNA transformation have been developed for several members of the genus *Phytophthora*, which includes many important phytopathogens. These eukaryotic microbes belong to the oomycete clade of the stramenopile (heterokont) group. Transformation of oomycetes was first achieved with the potato late blight pathogen *P. infestans,* and later extended to the soybean pathogen *P. sojae* and others (Judelson et al. 1991; Judelson et al. 1993a). The most common strategies for introducing plasmid DNA involve treating protoplasts with polyethylene glycol, or electroporating zoospores (Judelson and Ah-Fong 2009). Studies in *P. infestans* have shown that a plasmid usually integrates in tandem arrays at a single locus (Judelson 1993). Microprojectile bombardment and *Agrobacterium*-based methods for transformation have also been described (Cvitanich and Judelson 2003; Wu et al. 2016).

To date, the functions of about 40 *Phytophthora* genes have been tested through overexpression or homology-dependent silencing studies, including genes encoding effectors and diverse cellular proteins (Bos et al. 2010; rev. in Hardham and Blackman 2018). The latter method entails knocking-down mRNA levels of a target gene by expressing sense, antisense, or hairpin sequences from that gene in a stable transformant (Ah-Fong et al. 2008). The expression of hairpin sequences has also been referred to as DNA-directed RNAi by some researchers (Rice et al., 2005). Despite such advances, transformation remains challenging. Many researchers describe difficulty in obtaining sufficient numbers of transformants, transgene expression can be unstable, and the frequency of effective knock-downs can be low. It is thus common for studies of gene function to be based on a small number of transformants, sometimes as few as one. This is risky since phenotypes may be caused by unexpected events. About 20% of *P. infestans* transformants exhibit reduced fitness (Judelson et al. 1993b), which, based on studies of other organisms, might result from mutations caused by plasmid integration (Meng et al. 2007). Oomycetes are normally diploid with complex genomes in which gene-dense clusters are surrounded by repetitive DNA (Haas et al. 2009). For example, the 240 Mb genome of *P. infestans* contains about 18,000 genes and 74% high-copy sequences (Haas et al. 2009). Although the integration of plasmids into oomycete chromosomes is believed to involve nonhomologous recombination, no studies have described where plasmids integrate. It is unknown whether plasmid DNA inserts preferentially into gene-dense, gene-sparse, intragenic, or intergenic regions.

Although the mechanism of homology-based silencing has been studied in only a few cases, in *P. infestans* this has been shown to result in small RNA production

followed by transcriptional silencing and the formation of repressive chromatin (heterochromatin) at the target site (Ah-Fong et al. 2008; Judelson and Tani 2007; van West et al. 2008). Studies in plants and animals indicate that repressive chromatin can move along a chromosome, although its effect on endogenous genes is limited by barrier insulators or regulated by transcription factors (Elgin and Reuter 2013; Le et al. 2013). In contrast, transgenes or translocated native genes usually lack such protections and thus can become silenced by the *cis*-spreading of heterochromatin (Talbert and Henikoff 2006). It is thus reasonable to consider that heterochromatin may spread beyond the target of gene silencing in *Phytophthora*, causing a phenotype to be ascribed to the incorrect gene. We previously used the spreading phenomenon to our advantage by knocking-down a cluster of genes in the same metabolic pathway (Abrahamian et al. 2016). However, no study has examined how often silencing spreads in *cis* from a target to an unintended gene in any oomycete. Similarly, whether silencing affects off-targets elsewhere in the genome has remained unexplored.

The goal of this publication is to provide data to promote the wise use of transformation in oomycetes, although our findings are also relevant to other systems. We first describe the mapping of transgene integration sites in *P. infestans* using targeted DNA sequencing. Insertions occurred in both gene-rich and gene-poor regions, with a disproportionate fraction residing near the 5' ends of genes. We also present data from homology-dependent silencing studies of twelve genes to show how often silencing spreads from the intended locus. Genes spaced within 500 nt of the target were frequently co-silenced, but there were exceptions and co-silencing usually did not occur in all transformants. Off-target effects against unlinked genes appeared to be

infrequent. Thus, gene silencing and transformation can be reliable technologies but must be used with prudence.

RESULTS

Identifying locations of transgene insertions.

Transformants of *P. infestans* were selected from two prior studies, one focused on silencing its *INF1* elicitin gene and another on expressing the β-glucuronidase reporter (Ah-Fong et al. 2008; Judelson 1993). Transformants from the first study, denoted in this paper with the prefix S, were generated using a plasmid that expresses *INF1* sequences driven by the promoter from the *Ham34* gene of *Bremia lactucae* (Judelson et al. 1992) to trigger silencing. This plasmid also expresses *nptII* driven by a promoter from the *Hsp70* promoter of *B. lactucae* to confer G418 resistance. The second group of transformants, named with a G prefix, were obtained by a co-transformation experiment using *Eco*RI-linearized pTH209, which expresses *nptII* behind the *Hsp70* promoter, and *Eco*RI-linearized pHAMT35G, which expresses *uidA* (GUS) driven by the *Ham34* promoter. Prior studies showed that linearized plasmids usually ligate to each other, circularize, and integrate at a single locus (Judelson 1993).

To identify the plasmid insertion sites, genomic DNA was analyzed with the aid of targeted sequencing. Illumina libraries were enriched for plasmid-containing inserts by affinity purification using biotinylated 120-nt oligonucleotides, and sequenced to obtain 100-nt paired-end reads. The insertion sites were then mapped by a two-stage process. First, we detected paired-end reads where one read matched *P. infestans* genomic DNA and the other matched plasmid DNA. The approximate insertion site was then defined by the 3' end of the assembled *P. infestans* sequences. Since the average

insert size of the libraries was about 240-nt, the boundary between genomic and plasmid DNA calculated in this manner should be fairly close to the true insertion site. In the second stage of analysis, the precise junction was defined by searching for single reads that were chimeras of *P. infestans* and plasmid sequences, for example where 40-nt of a 100-nt read was of plasmid origin and the remaining 60-nt matched *P. infestans* DNA. There appeared to be insertions at a single site per transformant, including in the transformants obtained by cotransformation of two plasmids, which is consistent with our prior studies of *P. infestans* transformation (Judelson 1993).

The targeted sequencing approach allowed insertion sites to be identified in seven S and three G transformants. These sites are illustrated by the inverted triangles in Fig. 1. Eight of the sites were validated by polymerase chain reaction (PCR) using primers based on the left and right arms of each junction region (Fig. 2). In transformants G2 and G3, it was difficult to identify the precise insertion site since the apparent junction sequences contained highly conserved repetitive DNA; attempts to confirm the sites by PCR failed since the reactions yielded smears of bands, as shown for G3 in Fig. 2. BlastN and BlastX analysis of the repetitive sequences at the G2 junction against GenBank identified matches to reverse transcriptases, suggesting that the integration had occurred in a retroelement-like region. The sequence at the G3 junction lacked significant matches.

An eleventh insertion site (G4) was identified using whole-genome Illumina sequencing (Fig. 1). This involved analyzing ~10-fold sequence coverage of 12 progeny from a cross where one of the parents was a GUS-expressing transformant (Matson et al. 2015). Plasmid sequences were detected in five of the 12 progeny; the 5:7

158 segregation ratio is not significantly different (*P*=0.56) from what would be expected for
159 a single-site insertion. The junction sequence was identified by searching for reads
160 containing both plasmid and genomic DNA. The same junction was identified in multiple
161 progeny, supporting its authenticity.
162
163 **Genomic and biological context of insertions.**
164 Three of the integration events were in gene-dense regions and eight in gene-sparse
165 regions, although two of the latter (S1, S4) were within 1-kb of the 3' ends of genes (Fig.
166 1). There was no obvious correlation between plasmid copy number and the density of
167 genes near the insertion site. Each of the eleven events was on a unique supercontig in
168 the *P. infestans* assembly. None of the S transformants had integrated at the native
169 *INF1* locus, which matched sequences on the plasmid. Since the G2 and G3 insertions
170 were difficult to confirm due to repetitive DNA, the locations shown in Fig. 1 represent
171 one of 15 to 20 possible insertion sites. Nevertheless, each candidate region in G2 and
172 G3 was repeat-rich and gene-poor.
173 Three of the 11 integration events were predicted to affect the expression of a native
174 *P. infestans* gene. In S5 and G1 these were within the open reading frames of genes
175 PITG_05678 and PITG_06920, which encode a conserved protein of unknown function
176 and a Golgi transport protein, respectively. These insertions would thus result in
177 aberrant or dysfunctional proteins. In S7, the plasmid integrated near the 5' end of
178 PITG_14392, which encodes a ubiquitin protease. The insertion was 295-nt upstream of
179 the start codon and 270-nt upstream of the transcription start site, based on mapping
180 RNA-seq reads. The adjacency of the insertion to the regulatory region of PITG_14392

may influence that gene's expression.

The phenotypes of the transformants (right column in Fig. 1) were checked for an association with the nature of the integration event. There was a hint of a relationship between the site of insertion in the S strains and whether the native *INF1* gene had been silenced. Neither transformant in which the integration was in or near a gene (S5, S7) was silenced for *INF1*. This may be because homology-dependent silencing in *P. infestans* usually affects the expression of both the native gene and transgene (Ah-Fong et al. 2008; Van West et al. 1999), which may have been lethal if repressive chromatin had spread to any of the flanking genes. Although the number of insertion sites successfully mapped in G transformants was low (several failures in targeted sequencing are not described here), there was not an obvious correlation between phenotype and the site of integration. G1 and G4, which expressed the GUS-encoding transgene for more than two years, had integrations in gene-dense and gene-sparse regions, respectively. G2 and G3, which expressed GUS for about a month before losing activity, contained the insertion in a gene-sparse region. We previously showed that transformants that stop expressing GUS still maintain the gene, and this was confirmed by our sequencing data (Judelson and Whittaker 1995).

Detection of microhomology at many insertion sites.

The sequences of the confirmed junctions are shown in Fig. 3. In five cases, some conservation was observed between genomic and plasmid sequences at the site of recombination. This included an 8-nt region of identity (5'-GGAGCGTT) in transformant S1, a 3-nt region in S3 (AGC), and 2-nt regions in S7 (AA), G1 (CT), and G4 (GC).

Microhomology was not seen at the sites of the other events. In S2, one base at the junction did not match sequences in plasmid or genomic DNA and might be the product of DNA repair or a polymorphism arising during culture. The base composition of the 8-nt regions flanking the junctions was slightly skewed, averaging 27% A, 21% C, 34% G, and 18% T. Skewed base composition near transgene insertion sites has been reported previously in other species (Brunaud 2002).

Frequency of the longitudinal extension of gene silencing.

We examined 38 strains of *P. infestans* from silencing studies of twelve target genes to assess how often transcriptional silencing spread to neighboring loci. This used both newly generated silenced strains and those saved from prior projects (Table 1). Genes that flanked the silencing target were identified and their expression measured by reverse transcription-quantitative PCR (RT-qPCR) or genome-wide studies, *i.e.* RNA-seq. The flanking genes were identified using annotations in the *P. infestans* reference assembly (strain T30-4) and comparisons with an assembly of isolate 1306, which was the progenitor of the transformants. The results are presented in Figs. 4 and 5, where each graph represents a unique transformant.

The analysis of cases where the genes subjected to silencing were fairly far (>7 kb) from their neighbors led to the conclusion that silencing is unlikely to spread over many kilobases. For example, the gene silenced with a hairpin in Fig. 4A (PITG_11668) resided 10-kb from its left and right-most neighbors (PITG_11666 and PITG_11671), which maintained normal levels of expression. A similar outcome is shown in Fig. 4B, in which a hairpin against PITG_11664 did not lead to the repression of its flanking genes.

227 Analysis of the transformant in Fig. 4C, in which PITG_09198 was targeted, led to the
228 same conclusion. In this case, the hairpin had 98-99% nucleotide identity to seven
229 genes distributed within a 54-kb cluster. Each of the seven genes became co-silenced,
230 probably due to the direct effect of the hairpin. In contrast, genes on the left and right
231 flanks of the cluster (PITG_15172 and PITG_09196) and an intervening gene
232 (PITG_14821) maintained normal levels of expression. These transformants were
233 reported in a prior study (Gamboa-Melendez et al. 2013).

234 A situation where the flanking genes were close to the targeted locus is presented in
235 Fig. 4D. In this case, PITG_07059 was silenced in five transformants using a sense
236 construct (Fig. 4D1 to 4D5) and in one using a hairpin (Fig. 4D6). In the first three
237 transformants, normal mRNA levels were displayed by both neighboring genes, which
238 were 139 and 852-nt from PITG_07059. However, one or both neighbors were
239 repressed by about 50% in the transformants shown in Fig. 4D4, 4D5, and 4D6. This
240 was seen in two biological replicates. The divergent outcomes between strains indicate
241 that there is some randomness or transformant-specific variation in how silencing is
242 imposed.

243 A second example where the flanking genes are close to the targeted locus is shown
244 in Fig. 4E. These strains were obtained using a hairpin construct based on
245 PITG_01718. In both transformants, the knock-down effect did not spread to the right-
246 flanking gene, which was 400-nt from the target of the hairpin. The left-flanking gene
247 was expressed at normal levels in one transformant (Fig. 4E1) but was repressed by
248 about 50% in the other (Fig. 4E2). The partial knock-down of the left-flanking gene was
249 observed in two biological replicates.

Similar results were observed in transformants silenced with a hairpin from PITG_16100 (Fig. 4F). In this case, each flanking gene was about 400-nt from the targeted gene. In the four transformants in panels F1 to F4, both flanking genes were expressed at normal levels. However, the left neighbor was repressed by about 40% in the two transformants in panels F5 and F6. This was observed in two biological replicates.

A complex outcome was seen when transformants silenced for a clustered gene family was studied (Fig. 5G). These strains were previously used to show that silenced loci undergo heterochromatinization (Judelson and Tani 2007). The three genes in the cluster have about 80% overall nucleotide identity, with >95% identity within their middle and 3' ends. A full-length hairpin made from PITG_11238 was found to also knock-down PITG_11237 and PITG_11239 by nearly 100% (Fig. 5G1 and 5G2). A full-length hairpin based on PITG_11237 that strongly silenced its target partially knocked-down PITG_11238 and PITG_11239, but only by 50-75% (Fig. 5G3). In contrast, the genes flanking this cluster exhibited normal levels of transcription, including PITG_11236 which was 996-nt away and may share a promoter with PITG_11237. These results were confirmed in two biological replicates. Several processes may explain how silencing occurred within this gene family. Cluster-wide silencing may have been caused by the hairpin directly, or by broadcasting repressive chromatin from one of the genes. The partial knock-down of PITG_11238 and PITG_11239 in the strain in Fig. 5G3 might be due to incomplete repression within each nucleus or heterogeneous nuclear states resulting in variegated expression, similar to that observed in plants and animals (Timms et al. 2016).

A situation where silencing usually spread to the neighboring genes is shown in Fig. 5H. Panels H1 and H2 illustrate cases where a sense construct designed against PITG_13012 partially knocked-down flanking genes PITG_13011 and PITG_13013, which reside about 500-nt from the original target. These transformants were described in a prior report (Abrahamian et al. 2016). The spread of silencing appears to have been limited as the next-closest flanking genes were not affected. This was observed in three biological replicates. Interestingly, a sense construct based on PITG_13013 caused silencing to spread to PITG_13012 and PITG_13011 (Fig. 5H3). This suggests that repressive chromatin triggered by gene silencing can spread through multiple genes that are unrelated in sequence.

Another example of silencing spreading to adjacent genes is shown in Fig. 5I. Each of six strains in which homology-dependent silencing was used against PITG_03290 displayed strong down-regulation of flanking gene PITG_03291, which is 544-nt away. This occurred with both sense or hairpin constructs. This was observed in at least two biological replicates.

Our final example involves PITG_16104, which was silenced using a hairpin from the 5’ end of the gene (Fig. 5J1 to 5J3) and a hairpin from the 3' end (Fig. 5J4). These transformants have not been described previously. In each of the four transformants, the left-flanking gene exhibited normal expression even though it was only 666-nt from the targeted gene. Since the neighbor to the right, PITG_16105, was much farther from the target locus (3-kb) it was surprising to see that it was down-regulated by about 50% in each transformant. Interestingly, an analysis of the distribution of RNA-seq reads from isolate 1306 revealed the presence of a non-coding RNA between PITG_16104

296 and PITG_16105, which is labeled in the figure as ncRNA. This was consistently co-
297 silenced with PITG_16104. Therefore, it seems that a repressive chromatin state was
298 *cis*-propagated through the non-coding RNA locus, which had a mild influence on
299 PITG_16105 transcription.
300
301 **Off-target effects occurring *in trans* are infrequent**
302 We next focused on the type of off-target effect that in other organisms is
303 conventionally associated with gene silencing methods such as RNAi, which involves
304 the knock-down of genes with partial complementarity to the target. This was explored
305 using strains of *P. infestans* silenced for four of the genes described in the prior section.
306 Rules for predicting off-targets in plants and animals vary but include having >90%
307 identity within a 19-nt region, 11-nt of contiguous identical nucleotides, or partial identity
308 within about 15-nt provided that a seed region is conserved (Fellmann and Lowe 2014;
309 Jackson et al. 2006). Consequently, we defined potential off-target genes in *P. infestans*
310 as those having at least 11-nt of identity, 13 to 14-nt of identity with one mismatch, 15 to
311 19-nt of identity with up to two mismatches, or 20 to 21-nt of identity with up to three
312 mismatches to the silencing construct. Searches for complementarity examined exons,
313 introns, and 60-nt of extra bases at the 5' and 3' ends of each gene as potential
314 untranslated regions. Whether candidate off-targets were misregulated was assessed
315 by RNA-seq based on two biological replicates with a minimum of 25 million Illumina
316 reads each. We focused on genes exhibiting a two-fold or greater knockdown
317 compared to controls, since this threshold is commonly used to define short interfering
318 RNA (siRNA) off-targets in mammals (Caffrey et al. 2011; Ui-Tei et al. 2008).

319 Few, if any, down-regulated off-targets were detected in a strain silenced for
320 PITG_11664 with a hairpin (Fig. 6A). Based on sequence analysis, candidate off-targets
321 and non-targets of the hairpin numbered 3,560 and 10,308 genes, respectively. Only
322 one candidate off-target was down-regulated based on thresholds of $P \leq 0.01$ and 50%
323 repression compared to empty vector controls. The gene, PITG_11131, had 12-nt of
324 identity with the hairpin and an mRNA abundance of 49% compared to controls.
325 However, this seems to be a false positive. A similar fraction of predicted non-targets
326 (*i.e.* lacking complementarity to the silencing construct) were down-regulated, which is
327 suggestive of normal experimental noise. Also, no off-targets were down-regulated
328 significantly when a Benjamini-Hochberg multiple testing correction was applied.
329 Moreover, testing of an additional silenced strain showed that it expressed PITG_11131
330 at normal levels (89±24% of controls).
331 Little evidence for off-targets was also obtained from studying a transformant
332 silenced using a hairpin against PITG_09198 (Fig. 6B). Out of 3,158 candidate off-
333 targets, only two passed the 0.5× fold-change threshold with $P \leq 0.01$. One was
334 PITG_12405, which had a 11-nt match with the hairpin and was expressed at 46±3% of
335 the control level. However, this is probably a false positive since it was found to be
336 expressed at 120±11% of the control level in an additional silenced transformant.
337 PITG_06402 had a 17/19-nt match and was expressed at 47±30% of the control. In the
338 additional silenced transformant, it was expressed at 71±10% of control levels.
339 PITG_06402 could be a weak off-target, or could be regulated directly by PITG_09198
340 which encodes a transcription factor-like protein (Gamboa-Melendez et al. 2013). It
341 should be noted that three genes with 99% identity to PITG_09198 were silenced by the

342 PITG_09198 hairpin. These are not shown in Fig. 6B since they are considered targets
343 and not off-targets.

344 Little support for off-targets was also obtained by analysis of a transformant silenced
345 for the nitrate assimilation cluster, which includes genes PITG_13011, PITG_13012,
346 and PITG_13013, which encode a nitrate transporter, nitrate reductase, and nitrite
347 reductase, respectively (Fig. 6C). Only two genes out of 1,527 candidate off-targets
348 were potentially down-regulated according to the 0.5× fold-change and $P \leq 0.01$
349 thresholds. One was PITG_08758, which had 12-nt of complementarity with the sense
350 construct and was expressed at 45±3% of control levels. However, this is probably a
351 false positive based on the testing of additional biological replicates. The second off-
352 target candidate was PITG_12961, a gene that encodes a tRNA synthetase and which
353 had a 15/16 match with the hairpin and was expressed at 48±2% of the control. The low
354 expression of PITG_12961 persisted in the additional replicates, being 51±4% of control
355 levels. However, instead of being an off-target effect this seemed to be a consequence
356 of down-regulating the gene cluster. This is because PITG_12961 mRNA was 48±5% of
357 the control in a strain in which the cluster was silenced using a construct targeting
358 PITG_13013.

359 Poor support for off-target was also obtained through the analysis of a strain
360 silenced for PITG_07059 using a sense construct (Fig. 6D). PITG_07059 encodes a
361 transcription factor that regulates sporulation, which modulates the transcription of
362 several thousand genes (Leesutthiphonchai and Judelson 2018). The analysis was
363 consequently limited to genes that exhibited less than a two-fold change during a time-
364 course of sporulation in wild-type *P. infestans,* using RNA-seq data from samples

365 obtained in a previous study (Xiang and Judelson 2014). This left 1318 candidate off-
366 targets and 5177 non-targets for analysis. Nine candidate off-targets appeared to be
367 down-regulated by 25-50%. However, these were expressed at normal levels in an
368 additional silenced transformant. One interpretation is that the nine genes were false
369 positives due to normal experimental noise, since many predicted non-targets also
370 appeared to be differentially expressed. It is also possible that some of the nine genes
371 are authentic off-targets, but that the effect is random and not seen in all transformants.
372 We also studied whether genes with 21-mers that have the strongest matches to the
373 silencing construct might be off-targets. This is a simplistic approach, but in the absence
374 of rules for defining off-targets in oomycetes this is a strategy that some researchers
375 might currently use. The analysis yielded no evidence for off-target effects. For
376 example, in the transformant silenced for PITG_13012, the 37 genes having 19 or more
377 identical bases per 21-mer averaged 98% of the mRNA level of controls, with no
378 individual gene exhibiting <50% and only one having <75%. Similarly, in the
379 transformant silenced for PITG_09198 using a hairpin construct, the 24 genes having a
380 19/21 or greater match to the hairpin showed an average mRNA level of 97% versus
381 the controls, with no individual gene having <50% expression and only one being <75%.
382
383 **Transgenes are not always co-silenced with the target**
384 Initial studies of homology-dependent silencing in *Phytophthora* reported that sense,
385 antisense, and hairpin constructs became silenced along with their targets, based on
386 RNA blot analysis of a modest number of genes (Ah Fong and Judelson 2003; Gaulin et
387 al. 2002; Judelson and Tani 2007; Latijnhouwers and Govers 2003; Van West et al.

1999). Most subsequent research has not distinguished between the transgene and native gene when analyzing transformants from a gene silencing study. However, we recently reported a case where the target was strongly knocked-down while the transgene was strongly expressed (Leesutthiphonchai and Judelson 2018). To explore this in more detail, we examined 12 strains silenced strongly for five genes and used sequences in their 3' untranslated regions to differentiate between transcripts of the native gene and transgene. This was possible since each silencing construct used the transcriptional terminator from the *Ham34* gene of *Bremia lactucae* (Judelson et al. 1992).

The results indicated that continued expression of the transgene was not a rare event (Fig. 7). Transcripts from the partial-length sense construct used to silence PITG_07059 were detected in two of three transformants at 119 and 176%, respectively, of the level of expression of the native gene. The phenotypes of the transformants (reduced sporulation) were similar regardless of whether the transgene was persistently expressed (Leesutthiphonchai and Judelson 2018). Expression of the hairpin transcript used against PITG_09198 was detected in one of two PITG_09198-silenced transformants, at 42% of the level of the native gene. However, transgene mRNA was not detected in three PITG_11664-silenced strains, two PITG_11668-silenced strains, and two PITG_13012-silenced strains.

DISCUSSION

This study focused on the unintended effects of homology-dependent silencing experiments in *P. infestans* and the location of plasmid insertions into chromosomes.

We observed that the expansion of silencing to genes residing less than 500-nt from the target was common, while more distant loci usually escaped co-silencing. Protein-coding and non-coding transcription units were both susceptible to the *cis*-spreading phenomenon. This occurred regardless of whether the neighboring gene was at the 5' or 3' end of the target, or if the silencing vector encoded sense sequences or a hairpin designed to generate double-stranded DNA. We therefore recommend that gene silencing studies include analyses of flanking genes to ensure that inferences about biological function are reliable. Since widening of the silenced zone appeared to be a stochastic event, it should be possible in most studies to identify strains that escape *cis*-silencing through more extensive screening. In both gene silencing and overexpression experiments, it is also prudent to ensure that independent transformants yield similar phenotypes in order to exclude artifacts caused by insertions into unexpected genes.

An additional useful product of our research was the observation that transgenes were not always co-silenced with the hairpin or sense construct. Our survey of the literature indicates that many laboratories fail to distinguish between the native locus and transgene when screening transformants from a gene silencing study in *Phytophthora*. This can lead to the mistaken conclusion that strains are not silenced or that the knock-down is only partial. Adjusting RT-PCR screens such that one primer binds to the 3' UTR is a simple solution. Although the current annotation of the *P. infestans* genome does not include information on UTRs for most genes, this information can be obtained using RNA-seq data that we have deposited in public databases (Bioprojects NCBI PRJNA361417 and PRJNA407960 in NCBI).

It is possible that checking for the proximity of the gene of interest to other

transcription units may help guide the design of the silencing plasmid. The *cis*-spread of silencing might be reduced by using shorter sequences, or those farther from flanking genes. Smaller constructs might be less effective at triggering silencing, however. We previously showed that a 21-nt hairpin silenced 2% of transformants, compared to 31% with a 978-nt hairpin from the same gene (Judelson and Tani 2007). The logic of using larger fragments is that not all 21-mers are equally potent, so longer sequences would allow Dicer to generate pools of 21-mers, of which some may be more effective than others (Luo et al. 2007).

The multiplicity of siRNAs that are presumably generated by our larger sense and hairpin constructs may also contribute to our finding that few if any genes having partial complementarity exhibited off-target effects. In animal knock-downs, pooling siRNAs is believed to reduce sequence-specific off-target effects by lowering the concentration of each individual siRNA (Hannus et al. 2014). While checking *Phytophthora* databases for potential off-targets is a prudent practice, it appears that small regions of identity (*e.g.* a 19/21-nt match) between a gene and 500-nt silencing construct are insufficient to result in a knock-down, although our results do not exclude the possibility of minor effects. Off-target effects are probably also minimized by the fact that the silencing transgene usually becomes transcriptionally quiescent in stable transformants. However, some researchers have reported achieving transient knock-downs in *P. infestans* using dsRNA, and whether that causes off-target effects remains to be determined (Whisson et al. 2005).

Results from both sense and hairpin constructs were reported in the present study. We showed previously that hairpin constructs induced silencing three times more often

than sense or antisense constructs (Ah-Fong et al. 2008). That conclusion was based on studies of only two genes, but hairpins have also exhibited superior performance in plants (Wesley et al. 2001). Nevertheless, our laboratory has continued to test both types of constructs. The sense plasmid is often an intermediate in making the hairpin, and cloning a hairpin in *E. coli* is sometimes challenging. Therefore, time may be saved by initiating *P. infestans* transformations with a sense construct. A full-length sense plasmid also may yield both silenced and over-expressing transformants.

Analyses of transformants from our published experiments did not reveal cases where unrecognized silencing had spread to flanking genes, which might have negated our conclusions about the roles of the targeted genes. Luckily, most genes from those historical studies were located 1-kb or more from their neighbors, beyond the interval in which silencing normally spread. The first native gene that our laboratory silenced, *PiCdc14*, was not described in this paper since it is more than 100-kb from flanking genes (Ah Fong and Judelson 2003), but our conclusion that Cdc14 regulates sporulation was confirmed in several species by other researchers (Whisson et al. 2005; Zhao et al. 2011). Unlike *PiCdc14*, many genes studied by other groups using gene silencing have close neighbors, and the cellular roles of some of those targets may need to be re-examined. About two-thirds of *P. infestans* genes reside in gene-dense zones where the median intergenic distance is 435 nt (Roy et al. 2013). This is smaller than that of most eukaryotes, such as *Arabidopsis thaliana* where the median intergenic distance is 1.5 kb (Zhan et al. 2006). Consequently, the expansion of repressive chromatin caused by a gene silencing construct is probably more problematic in *Phytophthora* than most other eukaryotes.

Our understanding of the machinery that causes silencing and its *cis*-spread in oomycetes is limited. Homology-base silencing in *P. infestans* has been linked with the transient expression of small RNAs, which arrests transcription based on nuclear run-on assays (Ah-Fong et al. 2008; Judelson and Tani 2007; Van West et al. 1999). DNAse protection studies and tests of histone modification inhibitors have suggested that silencing heterochromatinizes the target (Judelson and Tani 2007; van West et al. 2008), although only a few target genes have been studied to date. *P. infestans* encodes the core components for RNA silencing, namely an RNA-dependent RNA polymerase, two Dicers, and five Argonautes (Asman et al. 2016). Studies in model systems have shown that siRNAs bound to Argonaute guide chromatin-modifying enzymes to complementary loci, leading to transcriptional silencing. Work in fission yeast indicated that siRNA-Argonaute complexes are recruited to their targets through interactions with nascent transcripts (Shimada et al. 2016). The *cis*-spread of silencing may thus occur along mRNA molecules. In plants, the signal involved in post-transcriptional silencing was shown to extend 3'→5' and 5'→3' for up to 300 and 1000-nt, respectively (Petersen and Albrechtsen 2005; Vaistij et al. 2002). These distances resemble the zone susceptible to the spread of transcriptional silencing in *P. infestans.* Another mechanism for the *cis*-spread of silencing might involve propagating repressive histone marks. In mammals, histone methyltransferases G9a and GLP forms a complex that binds H3K9me, which allows the enzymes to read their own marks and spread the modification (Shinkai and Tachibana 2011).

Our data on plasmid integration sites stress the importance of drawing conclusions about gene function from studies of multiple transformants. Three of the eleven mapped

503 insertions (27%) were potentially mutagenic since they were within 300-nt of the start
504 site of *P. infestans* genes, including within coding sequences. Transformants bearing
505 such insertions might be reduced in fitness due to haploid insufficiency. How often this
506 would occur in an oomycete is a matter of conjecture, but about 15% of human genes
507 are believed to be lethal in a hemizygous state (Lek et al. 2016). There are also
508 precedents of insertions reducing fitness (Durkin et al. 2001). In *Phytophthora*, a single
509 insertion might alter virulence since effectors are often present in a hemizygous state
510 (Jiang et al. 2006).

511 The frequency of insertions near the start site of genes in our *P. infestans*
512 transformants occurred more than expected by random chance, as this space
513 represents only 4.3% of the genome. Studies in *Saccharomyces cerevisiae* showed that
514 plasmid integration occurred more easily in regions with low nucleosome occupancy,
515 which is common at the 5' ends of genes (Aslankoohi et al. 2012; Jiang and Pugh
516 2009). Disproportionate insertions into this area were also described for foreign DNA
517 and transposons in mouse cells, and of foreign DNA in *A. thaliana* (Yan et al. 2013;
518 Yant et al. 2005). The latter study also identified microhomology at recombination sites,
519 as we found in *P. infestans.*

520 Gene editing systems such as CRISPR/Cas9 provide an alternative to gene
521 silencing. This was recently adapted to *P. capsici, P. palmivora,* and *P. sojae* but has
522 not yet succeeded with *P. infestans* (Gumtow et al. 2018; Miao et al. 2018; van den
523 Hoogen and Govers 2018). Nevertheless, the silencing method may be advantageous
524 in some situations. For example, an entire gene family may be silenced by a single
525 construct (Grenville-Briggs et al. 2008; Judelson and Tani 2007). Optimizing both tools

for functional genomics would benefit studies of oomycetes.

MATERIALS AND METHODS

***P. infestans* transformation.** All transformants were obtained using isolate 1306, which is a diploid strain isolated from a tomato field in California, USA. Strains for insertion analysis were obtained using plasmids pHP4 (7.8-kb), pTH209 (4.8-kb), and pHAMT35G (6.0-kb). The latter two were cotransformed into *P. infestans* as linearized DNA, which is normally ligated together and circularized *in vivo* by *P. infestans* prior to chromosomal integration. Transformants were maintained on media containing 5-10 µg/ml G418 prior to DNA or RNA extraction. DNA blot analysis, measurements of GUS activity, and protein and RNA-based assessments of *INF1* silencing in strains used for insertion analysis were described previously (Judelson and Ah-Fong 2009).

Gene silencing experiments used sense or hairpin constructs expressed from the *Ham34* promoter (Table 1). The isolation of some of these were described previously as noted in Results. Others were generated using pSTORA (Judelson and Ah-Fong 2009) or a version modified to include additional cloning sites (pSTORAV). Strains were defined as silenced if the mRNA level of the target gene in RT-qPCR was <25% that of wild-type, based on three biological replicates of wild-type and the putative silenced strain.

Transformants were obtained using a modification of our original protoplast method (Judelson et al. 1991). Seed cultures for protoplasting were initiated by spreading about 10^4 sporangia per 150-mm plate of rye-sucrose media containing 1.5% agar, typically using 15 plates per experiment. After about 10 d at 18°C in the dark, 15 ml of room

549 temperature water were spread on each plate, and sporangia were rubbed from the
550 plate using a flamed bent glass rod. The liquid from the plates was decanted into a 1 l
551 flask, and hyphal fragments removed by filtering through 50 µm nylon mesh. An equal
552 amount of clarified amended lima bean media (Bruck et al. 1980) was then added, and
553 additional media (and an equal volume of water) were added to set the sporangia
554 concentration to 4×10^5 per ml. The media was then distributed to 1 l flasks, with 150 ml
555 per flask. After 24-36 hr of stationary growth at 18°C, the germinated sporangia had
556 formed a thin mat on the bottom of each flask. These were detached from the glass by
557 gentle swirling, and harvested by pouring the culture through 50 µm mesh and rinsed
558 with protoplasting buffer (PB; 0.4M mannitol, 20 mM KCl, 20mM MES pH 5.7, 10mM
559 $CaCl_2$). This wash was performed by picking up the hyphae from the mesh with bent
560 forceps, placing the hyphae in a 50 ml tube containing PB, and collecting the hyphae
561 again on the nylon mesh. For every ml of packed germlings, 3 ml of PB was added
562 containing 5 mg/ml of filter-sterilized cellulase (Sigma, from *Trichoderma reesi)* and 10
563 mg/ml β-glucanase; the latter was either Vinoflow NCE (Novozyme) or Extralyse
564 (Laffort). The tube was then shaken gently (40 rpm) on an orbital shaker. During this
565 incubation, 30 µg DNA (in 40 µl, volume adjusted as needed with water) was mixed in a
566 polystyrene tube with 60 µl Lipofectin (Thermo Fisher). When cell wall digestion was
567 about 90% complete based on microscopic examination, typically about 20-25 min, the
568 protoplast mixture was passed through Miracloth or 50 µm mesh, and pelleted in a
569 swinging bucket centrifuge at 700 ×*g* at room temperature. The pellet was resuspended
570 gently in 30 ml of PB and respun. The pellet was then resuspended in 15 ml PB, and 15
571 ml of MT (1 M mannitol, 10 mM Tris pH 7.5) was added. After another round of

572 centrifugation, the pellet was resuspended in 30 ml MT plus 10 mM $CaCl_2$, respun, and
573 resuspended at about 2.5×10^7 protoplasts per ml based on a concentration determined
574 before the final spin. The DNA was then added to 0.7 ml of the protoplast mixture in a
575 16 ml polystyrene tube with gentle mixing by inversion or gentle pipetting up-and-down.
576 After 4 min, 0.7 ml of 50% polyethylene glycol MW 3350 containing 25 mM $CaCl_2$ and
577 10 mM Tris pH 7.5 was added slowly, by pipetting the fluid down the side of the tube
578 while rotating the tube. After 4 min, 2 ml of clarified rye-sucrose broth containing 1 M
579 mannitol (rye-mannitol) at room temperature was added, the tube was inverted, and
580 after 1 min an additional 4 ml of rye-mannitol was added. After 1 min, the contents of
581 the tube were poured into 25 ml of additional rye-mannitol (in a plastic petri plate or a
582 50-ml tube) and the material incubated for 20-36 hr at 18°C. The regenerated tissue
583 was then spun at 1000 ×*g* for 5 min. The pellet was then resuspended in about 1.5 ml of
584 media containing mannitol with the aid of agitation using a 1-ml pipette, and spread on
585 rye-sucrose agar plates containing 7 µg/ml G418. These plates typically were amended
586 with 40 units/ml nystatin and 25 µg/ml penicillin G (or other antibacterial). Colonies
587 typically appeared after 7 d at 18°C.
588
589 **Targeted sequencing.** Library construction and target enrichment followed the protocol
590 described in Faircloth et al. (Faircloth et al. 2012) and Smith et al. (Smith et al. 2014),
591 which uses the solution-based probe capture method developed by Gnirke (Gnirke et al.
592 2009). Genomic DNA was sheared, size-selected to 200-500 nt, and used to prepare
593 sequence-tagged libraries using the Illumina Truseq kit with barcoded adapters. This
594 was subjected to probe capture using 597 biotinylated 120-mers that had been

595 designed to achieve three-fold coverage against each plasmid; for transformants
596 obtained using two plasmids, a pool of 120-mers from both plasmids were used. Since
597 these must be restricted to one strand of the plasmid to prevent self-annealing during
598 the enrichment procedure, only one side of the plasmid insertion is expected to be
599 mapped unless the plasmid underwent rearrangement in *P. infestans.* After hybridizing
600 the library to the 120-mers, complexes were purified with streptavidin-coated beads and
601 eluted. After quantifying the enriched libraries, they were pooled and 100-nt paired-end
602 reads obtained using an Illumina 2500 instrument by a commercial provider. The
603 libraries yielded from 296,241 to 6,539,323 clean reads, of which 4 to 45% matched a
604 plasmid. This was calculated to represent between 80 to 400-fold enrichment
605 considering the plasmid copy number of each transformant.
606 After removing adapters and quality trimming, GSNAP was used to map the reads to
607 the *P. infestans* genome and plasmids. This used an assembly of isolate 1306
608 developed using Pacific Biosciences and Illumina reads (Pan et al. 2018). The matches
609 were then searched to identify read pairs where one read mapped to the genome and
610 its pair mapped to a plasmid. This was verified using BLASTN. To avoid spurious
611 results, insertions were defined when a minimum of 20 such sequences were identified.
612 The approximate location of the insertion was then identified by assembling the
613 genomic portion of those reads. The coordinates were then converted into the locations
614 in the public assembly of *P. infestans* strain T30-4 using the BLASTN tool at
615 fungidb.org. Gene locations, and in some cases transcription start sites, were validated
616 by visualizing the distribution of RNA-seq reads using Integrated Genome Viewer
617 (Thorvaldsdottir et al. 2013). The reads used have been deposited in NCBI as

Bioproject PRJNA361417.

Polymerase chain reaction. Validation of insertion sites was performed using 20 ng of template DNA, primers designed to amplify junction fragments of 90-120 nt, and a program of 35 cycles of 94°C for 30 sec, annealing temperatures of 52°C to 58°C depending on the primer for 30 sec, and 72°C for 30 sec. RT-qPCR used primers designed to amplify fragments of 100-150 nt from the 3' ends of the target genes. RNA for RT-qPCR was isolated using the Sigma kit for plant RNA, DNase-treated, and cDNA synthesized using the Maxima (Thermo) First-Strand RT-PCR kit. Primers were tested using a dilution series of template and accepted if efficiencies were above 94%. Amplifications were performed using a Bio-Rad iCycler CFX Connect system using the Dynamo SYBR Green qPCR kit (Thermo) with the following program: 95°C for 15 min, followed by 40 cycles of 94°C for 30 sec, 52 to 58°C for 30 sec depending on the primer, and 72°C for 30 sec. Controls without reverse transcriptase and melt curves were included to help verify the reliability of the amplification. Expression levels were calculated using the $\Delta\Delta C_T$ method, using a constitutive gene (PITG_09862; Niu et al. 2018) as a control with at least two biological replicates, with three technical replicates each.

Analyses of flanking genes. To study the spread of silencing, we examined the annotation of the *P. infestans* reference genome, which was generated by Sanger sequencing of strain T30-4 (Haas et al. 2009). Some predicted flanking genes appeared to be unexpressed in isolate 1306 or false annotations based on a lack of RNA-seq

reads from hyphae grown on rich and defined media, media containing fungicides, sporulating hyphae, sporangia, sporangia chilled to induce zoospores, zoospore cysts, mating cultures, and early and late timepoints of potato leaf, tomato leaf, and potato tuber infection (from NCBI Bioprojects PRJNA361417, PRJNA407960, and unpublished data). When a gene was deemed to be missing or unexpressed in isolate 1306, the next-closest gene was chosen as the flanking locus. If the region flanking the gene of interest in the T30-4 assembly lacked annotated genes, or contained gaps, we validated the assembly, or filled gaps, using the 1306 assembly (Pan et al. 2018). The filled gaps did not appear to contain genes, except in the case of PITG_09199.

Expression analysis of the targeted genes and their neighbors were usually obtained by RT-qPCR. However, data for PITG_09198, PITG_11664, and PITG_11668 were obtained by RNA-seq with the workflow described previously (Ah-Fong et al. 2017a). In brief, a minimum of 25 million reads were obtained per biological replicate, with a minimum of two biological replicates. Reads were aligned and mapped to gene models using Bowtie version 2.2.5 and Tophat version 2.0.14, allowing for 1 mismatch. Expression and differential expression calls were made with edgeR using TMM normalization, and a generalized linear model.

Genome-wide analysis of potential off-targets. Candidate off-targets were identified using a modified version of BLASTN against a database of the predicted nascent transcripts of *P. infestans,* with 60 bases added to each end of the predicted gene model to represent UTRs. Searches were performed using expect thresholds of 2000, a word size of 4, and match and mismatch scores of 3 and 2, respectively. Parameters

were modified to exclude gapped alignments and low-complexity masking. Expression levels were then studied using RNA-seq or RT-qPCR as described above, except that data were trimmed using a CPM threshold of 2.0 to exclude unreliable signals.

Statement of author contributions

H.S.J. designed the study. All authors generated and analyzed experimental data. All authors participated in the writing of the paper.

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917

FIGURE LEGENDS

Fig. 1. Plasmid integration sites in *P. infestans* transformants. Insertion sites of the S and G transformants are marked by inverted triangles, with numbers above each triangle denoting the position on supercontigs from the assembly of strain T30-4 (sc). Blue arrows represent the coding sequences of genes, with three genes mentioned in Results labeled with the PITG prefix. The columns on the right indicate plasmid copy numbers and phenotypes. For S strains, the latter are INF-silenced (Sil+) and non-silenced (Sil-), and for G strains these are GUS-expressing (GUS+) or strains in which GUS activity was short-lived (GUS-). The insertion in S3 was in a region absent from the T30-4 assembly, but was in an assembly of strain 1306.

Fig. 2. PCR validation of insertion sites. A, Amplification using primers flanking the plasmid-chromosome junction. As shown in the diagram, primers P1 and P2 are within genomic and plasmidic DNA to the left and right of the bioinformatically predicted recombination site, respectively. The top row of images shows reactions using transformant DNA with transformant-specific primers. The bottom row of images are negative controls using each primer set against DNA from progenitor strain 1306. The samples match the transformants in Fig. 1. MW is a DNA ladder. The right panels came from a separate gel; the dashed lines indicate where some lanes were reordered or blank lanes were excised. **B,** Amplification of genomic DNA using primers against PITG_09862, which serves as a positive control for DNA quality. NC is a no-template control.

Fig. 3. Sequences flanking integration sites. The top, middle, and bottom lines of each alignment correspond to the genomic from each transformant (*e.g.* S1), the plasmid (PLAS), and the progenitor strain (1306). Bases in red and green indicate sequences in the transformant that originate from genomic and plasmid DNA, respectively. The breakpoints between genomic and plasmid DNA are marked by boxes and inverted triangles. The boxes indicate regions of microhomology between plasmid and genomic DNA.

Fig. 4. Expression levels of genes flanking the silenced target. Indicated in each panel are the genes targeted for silencing (black bars) and that closest genes that flank the target (white bars). The Y-axis indicates the mRNA level of each gene relative to wild-type. The X-axis is drawn to scale, and portrays the size of each gene and its distance to its neighbors. Distances to flanking genes are denoted in italics. Each gene is labeled with its database number (trimmed of the PITG prefix) and an arrow that indicates the orientation of transcription. The grey bars indicate the sequences used in the silencing vector as sense (s) or hairpin (hp) constructs. The locations of genes in Fig. 4C were determined using a long read-based assembly of isolate 1306 that allowed contigs used in the Broad Institute's assembly of *P. infestans* strain T30-4 to be joined and reordered. Genes in 1306 that were absent in the T30-4 assembly are marked as BZP.

Fig. 5. Expression levels of genes flanking the silenced target. This is a continuation of Fig. 4.

964

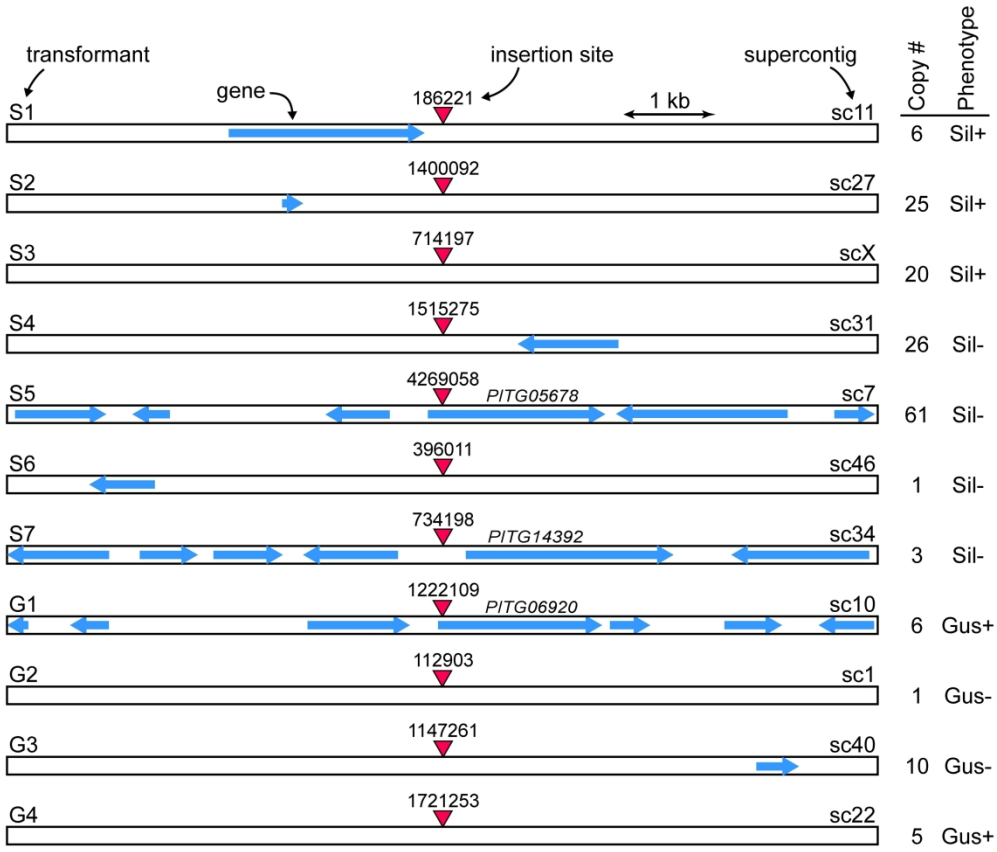
965 **Fig. 6. Effect of silencing on candidate off-target genes.** mRNA levels were
966 measured by RNA-seq in transformants silenced with hairpin constructs against
967 PITG_11664 **(A)** or PITG_09198 **(B)**, or sense constructs against PITG_13012 **(C)** or
968 PITG_07059 **(D)**. Candidates for off-targets based on partial sequence complementarity
969 are in blue. Other genes (candidate non-targets) are in orange. The *X*-axis indicates the
970 mRNA level in the silenced strains compared to the non-silenced control. The extent of
971 silencing of the targeted gene is indicated in the lower left-hand corner of each panel.

972

973 **Fig. 7. Expression of the silencing transgene in transformants.** Indicated are mRNA
974 levels in wild-type *P. infestans* (WT) and 12 transformants silenced using constructs
975 targeting five *P. infestans* genes; strains silenced using the same plasmid are
976 distinguished by a, b, c following the five-digit identifier of the targeted gene. mRNA
977 levels of the native gene (white bars) and transgene (black bars) are expressed relative
978 to the level of the targeted gene in wild-type. Data are based on RNA-seq and RT-
979 qPCR analysis.

980

981



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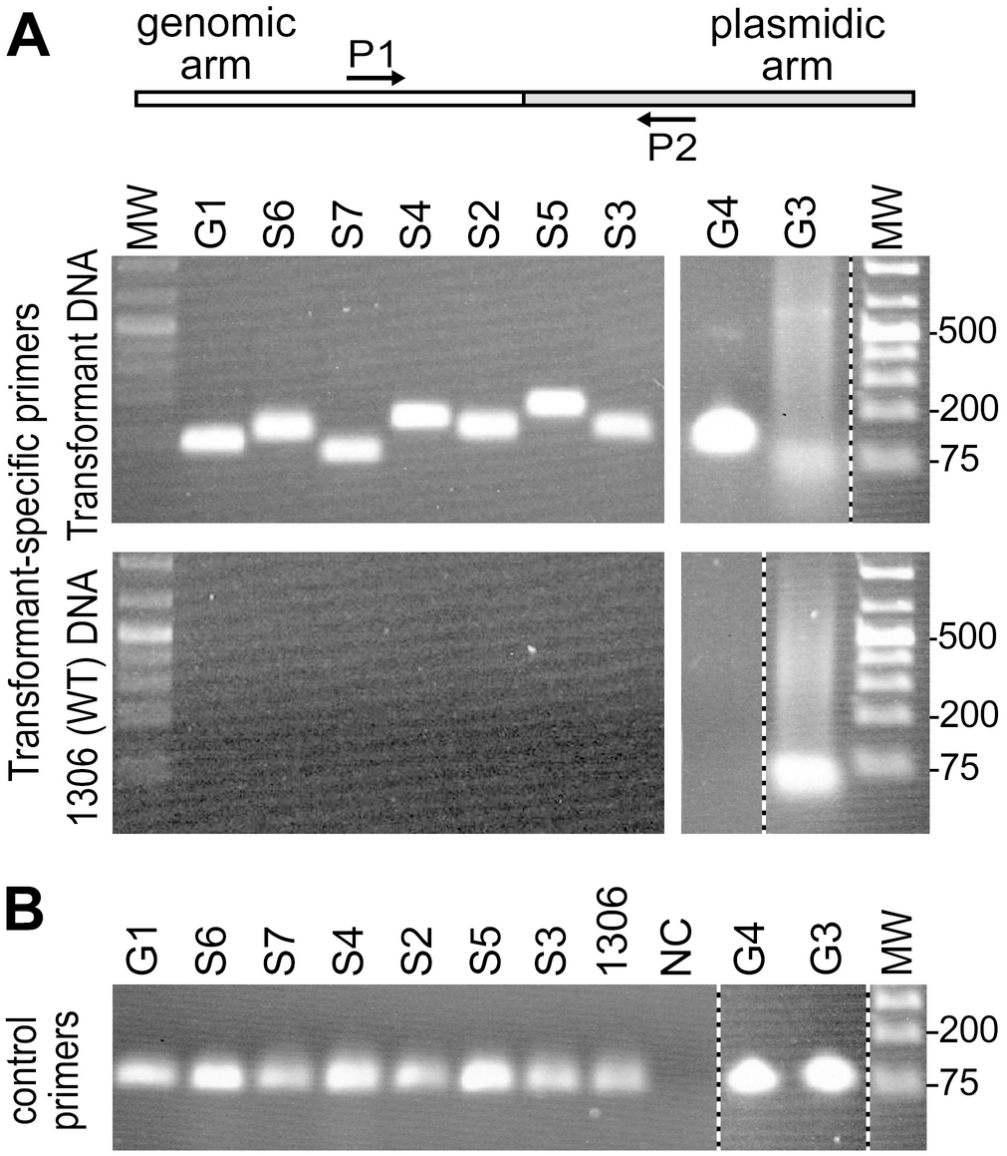

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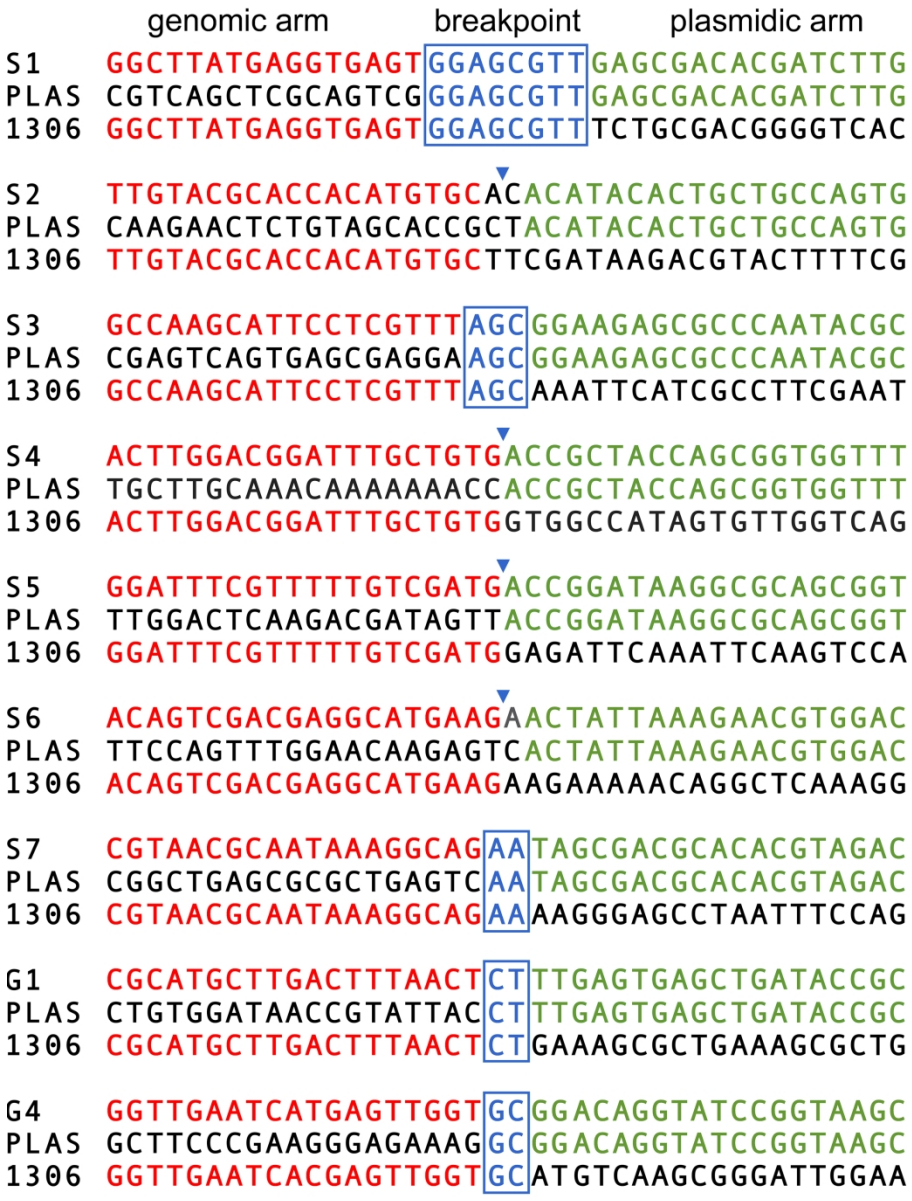

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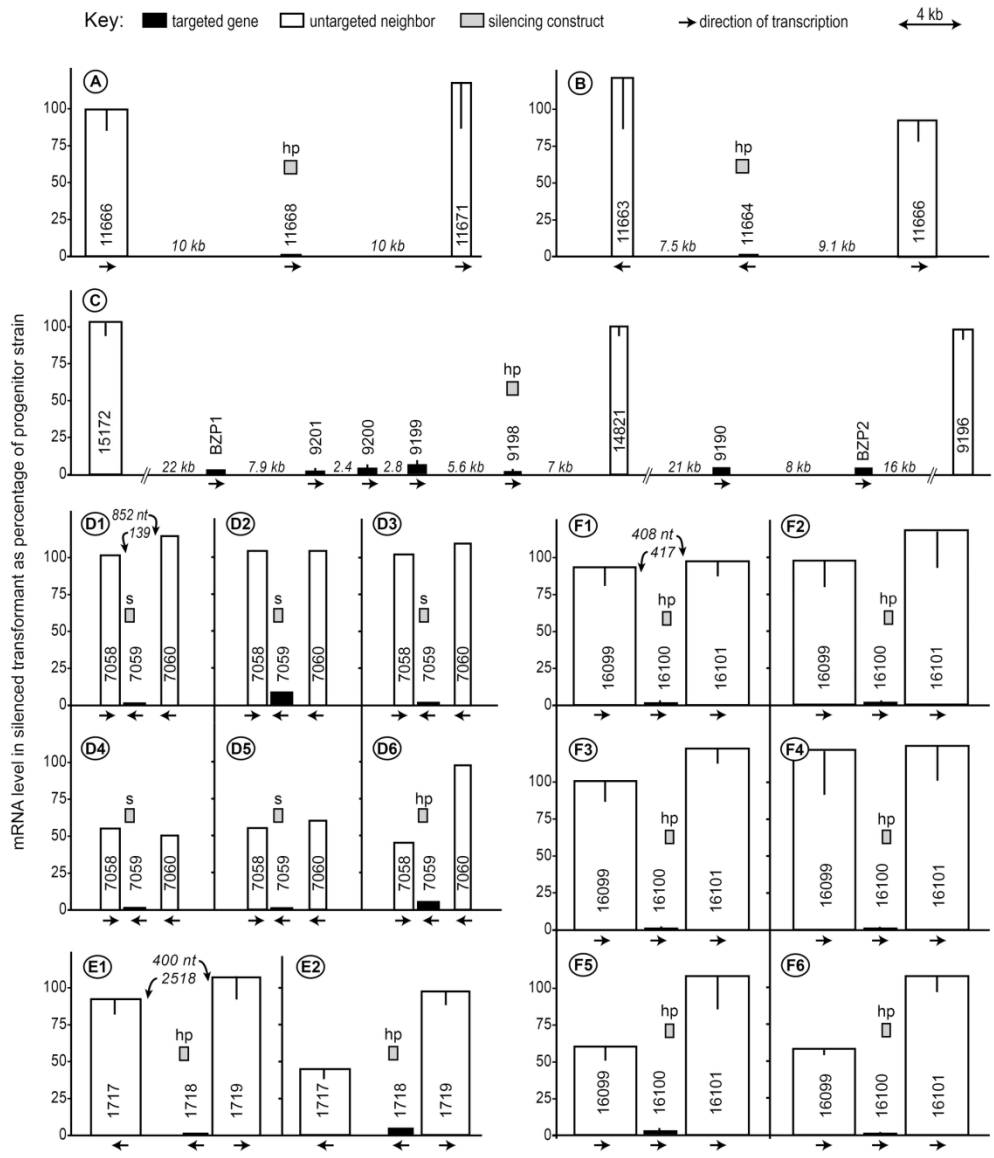

Fig. 4. Expression levels of genes flanking the silenced target. Indicated in each panel are the genes targeted for silencing (black bars) and that closest genes that flank the target (white bars). The Y-axis indicates the mRNA level of each gene relative to wild-type. The X-axis is drawn to scale, and portrays the size of each gene and its distance to its neighbors. Distances to flanking genes are denoted in italics. Each gene is labeled with its database number (trimmed of the PITG prefix) and an arrow that indicates the orientation of transcription. The grey bars indicate the sequences used in the silencing vector as sense (s) or hairpin (hp) constructs. The locations of genes in Fig. 4C were determined using a long read-based assembly of isolate 1306 that allowed contigs used in the Broad Institute's assembly of P. infestans strain T30-4 to be joined and reordered. Genes in 1306 that were absent in the T30-4 assembly are marked as BZP.

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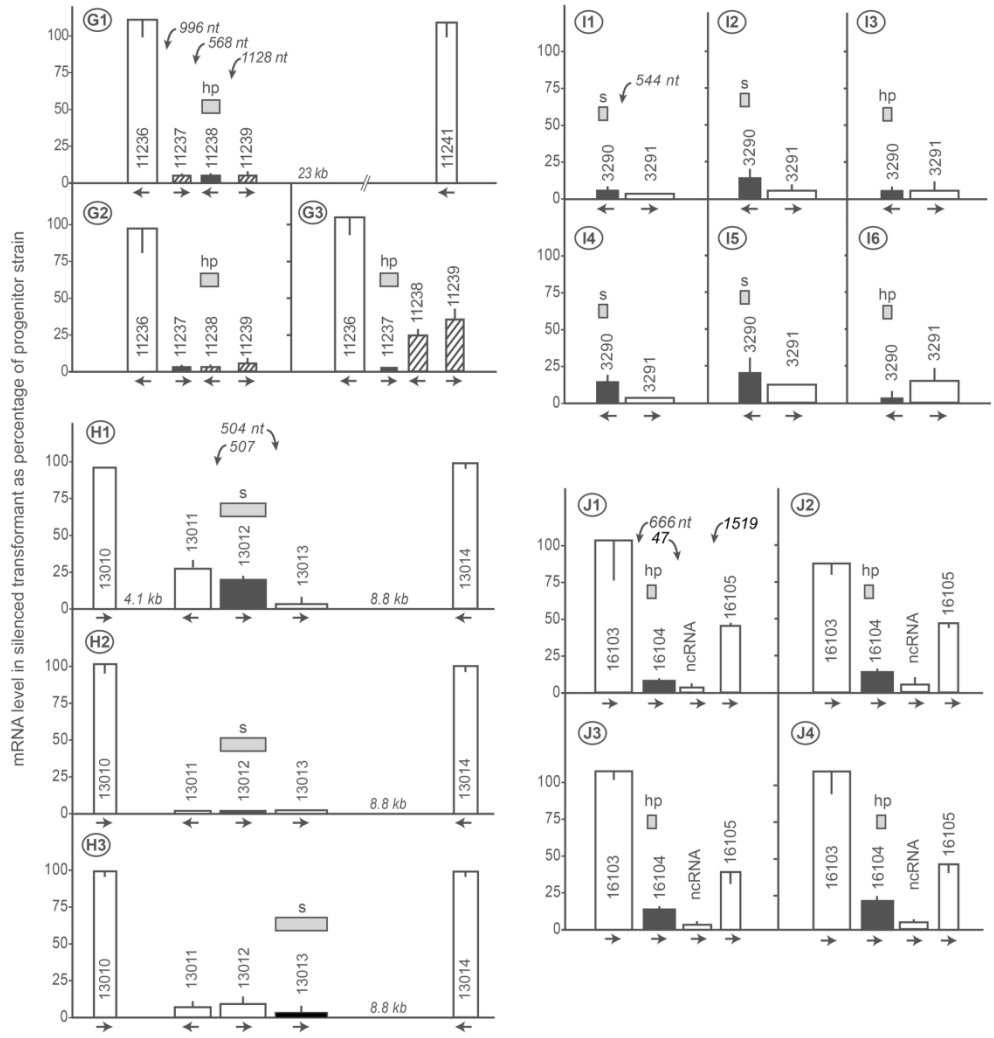

Fig. 5. Expression levels of genes flanking the silenced target. This is a continuation of Fig. 4.

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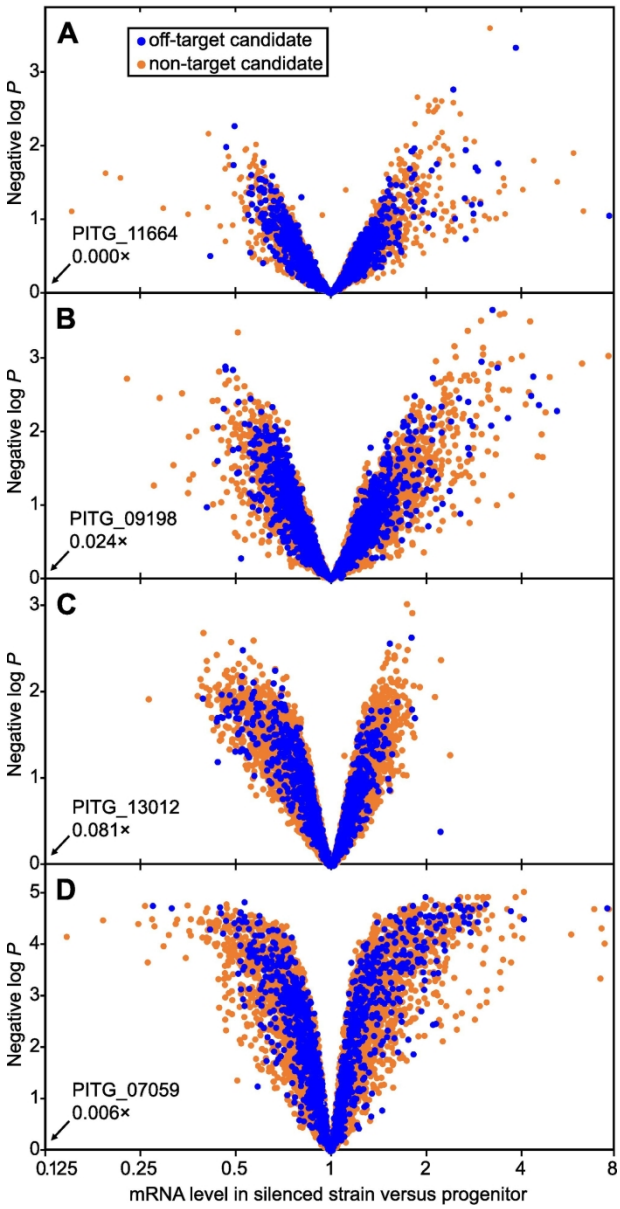

Fig. 6. Effect of silencing on candidate off-target genes. mRNA levels were measured by RNA-seq in transformants silenced with hairpin constructs against PITG_11664 (A) or PITG_09198 (B), or sense constructs against PITG_13012 (C) or PITG_07059 (D). Candidates for off-targets based on partial sequence complementarity are in blue. Other genes (candidate non-targets) are in orange. The X-axis indicates the mRNA level in the silenced strains compared to the non-silenced control. The extent of silencing of the targeted gene is indicated in the lower left-hand corner of each panel.

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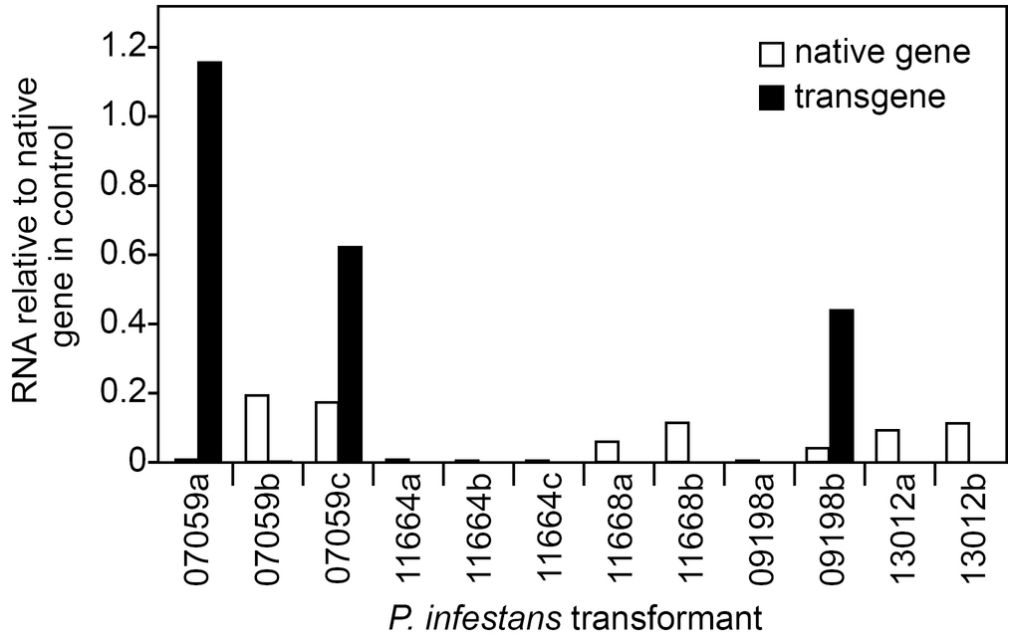

Fig. 7. Expression of the silencing transgene in transformants. Indicated are mRNA levels in wild-type P. infestans (WT) and 12 transformants silenced using constructs targeting five P. infestans genes; strains silenced using the same plasmid are distinguished by a, b, c following the five-digit identifier of the targeted gene. mRNA levels of the native gene (white bars) and transgene (black bars) are expressed relative to the level of the targeted gene in wild-type. Data are based on RNA-seq and RT-qPCR analysis.

85x54mm (300 x 300 DPI)

Table 1. Transformants used in this study

Gene	Configuration in silencing construct	Full-length coding sequence (nt)	Portion in silencing construct (nt)	Source of transformant
PITG_01718	hairpin	1707	517-1016	this study
PITG_03290	hairpin	1638	1063-1556	this study
PITG_03290	sense	1638	1063-1556	this study
PITG_07059	hairpin	1299	718-1196	this study
PITG_07059	sense	1299	718-1196	this study, Leesutthiphonchai and Judelson 2018
PITG_09198	hairpin	1053	255-833	Gamboa-Melendez et al. 2013
PITG_11237	hairpin	978	1-978	Judelson and Tani 2007
PITG_11238	hairpin	1023	1-1023	Judelson and Tani 2007
PITG_11664	hairpin	1044	1-546	Gamboa-Melendez et al. 2013
PITG_11668	hairpin	1101	200-700	Gamboa-Melendez et al. 2013
PITG_12551	hairpin	376	1-376	Ah-Fong et al. 2018
PITG_13012	sense	2709	1-2709	Abrahamian et al. 2016
PITG_13013	sense	3141	1-3141	this study
PITG_16100	hairpin	2085	1129-1608	this study
PITG_16104	hairpin	1971	13-512	this study
PITG_16104	hairpin	1971	897-1386	this study
uidA (GUS)	N.A.	1808	N.A.	Judelson 1993, Matson et al. 2015

Genes PITG_12551 *(INF1)* and *uidA* (β-glucuronidase) were used to obtain the "S" and "G" transformants shown in Fig. 1. As described in Materials and Methods, the G transformants were obtained by cotransformation with a plasmid encoding *nptII*. The other genes were used as targets for the silencing experiments shown in Figs. 4 and 5. N.A., not applicable