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Limitations of transcriptome-based prediction of pathogenicity genes in the plant pathogen *Leptosphaeria maculans*

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ABSTRACT

Identification of pathogenicity determinants in *Leptosphaeria maculans*, a major cause of disease of oilseed crops, has been a focus of research for many years. A wealth of gene expression information from RNA sequencing promises to illuminate the mechanisms by which the fungus is able to cause blackleg disease. However, to date, no studies have tested the hypothesis that high gene transcript levels during infection correlate with importance to disease progression. In this study we use CRISPR-Cas9 to disrupt 11 genes that are highly expressed during the early stages of disease and show that none of these genes are crucial for fungal pathogenicity on *Brassica napus*. This finding suggests that in order understand the pathogenicity of this fungus more sophisticated techniques than simple expression analysis will need to be employed.

Keywords: blackleg disease, CRISPR-Cas9, Dothideomycete, RNA-seq

Topic: gene expression, gene disruption, host-pathogen interactions

Issue Section: Pathogens and pathogenicity

INTRODUCTION

Leptosphaeria maculans is a major pathogen of canola (*Brassica napus*) and is endemic in many parts of the world (Fitt *et al*. 2006). This fungus initially infects the cotyledons and leaves of young plants then moves down to the base of the stem where it causes a canker that restricts nutrient flow and reduces structural integrity (Fitt *et al*. 2006, Hammond *et al*. 1985). In addition to avoidance of inoculum through farming practises, current management relies on a combination of resistance (*R*) genes in cultivars and fungicide application, without which the industry would not be viable (West *et al*. 2001). Alarmingly, both of these approaches are vulnerable to the development of resistance by the fungus. The "breakdown" of *R*-gene mediated resistance has occurred serval times in the past. This includes the rapid breakdown of *LepR3* in Australia in the early 2000s, which led to 90% yield losses and cost up to 10 AUD million dollars (Sprague *et al*. 2006), *Rlm1* in France between 1996 and 1999 (Rouxel *et al*. 2003), and more recently evidence that a breakdown of cultivar Hyola50 resistance on the Eyre Peninsula in Australia, which was ameliorated through the recommendation to sow cultivars with alternative *R* genes, which saved a predicted 13 million AUD dollars (Van de Wouw *et al*. 2014). There are signs that fungicides, that have become increasingly common in the industry, may also be vulnerable in light of recent reports of *L. maculans* isolates resistant to one of the most commonly used fungicides fluquinconazole (Van de Wouw *et al*. 2017). The development of new fungicides targeted to specific fungal proteins required for disease is a promising, yet unrealised, solution to this problem (Acero *et al*. 2011).

The identification of fungicide targets in *L. maculans* has thus been a focus of research and has largely centered around the identification of pathogenicity genes, defined as genes which

74 are essential for disease development. Unfortunately, despite decades of research only nine
75 such genes have been discovered whose deletion or silencing causes a substantial reduction in
76 pathogenicity (reviewed in Urquhart and Idnurm 2017). This slow rate of gene discovery may
77 in part be explained by the difficultly associated with making targeted gene disruptions in this
78 species via homologous recombination, which typically requires the cloning of at least 7 kb
79 of homologous DNA and then screening hundreds of transformants (Gardiner and Howlett
80 2004, Idnurm *et al*. 2003, Wilson *et al*. 2002). However, the development of CRISPR-Cas9
81 gene editing system in *L. maculans* could provide a more convenient system of gene
82 disruption (Idnurm *et al*. 2017), opening up new possibilities for the study of gene function in
83 this plant pathogen.
84
85 Another research focus in *L. maculans* has been gene expression profiling during infection
86 using RNA-sequencing based approaches (Gervais *et al*. 2017, Haddadi *et al*. 2016, Lowe *et*
87 *al*. 2014, Sonah *et al*. 2016). RNA-sequencing transcriptomics have also been extensively
88 used in a diverse selection of species, including economically-important fungal pathogens
89 (Naidoo *et al*. 2018). Such RNA-seq studies aim to identify those genes that are highly
90 expressed during host infection compared to in vitro culture, based on the hypothesis that
91 such genes will be required by the fungus to cause disease. Thus, these sets of up-regulated
92 genes represent a priority direction to explore as fungicide targets. However, to date, no
93 studies have tested this hypothesis in *L. maculans*. To address this fundamental gap, we
94 selected *L. maculans* genes strongly up-regulated during infection of canola cotyledons and
95 disrupted 11 genes using the CRISPR-Cas9 system. We found that all 11 strains with the
96 gene mutations were still able to cause disease on canola cotyledons, a finding that has
97 important implications for the interpretation and use of RNA-seq data in this species – and

likely other pathogenic fungi – in relation to the ongoing efforts to identify new fungicide targets.

MATERIALS AND METHODS

Analysis of RNA-sequencing data

Previously generated RNA-sequencing data were examined to identify genes up-regulated during the early stages of cotyledon infection, as well as to examine the expression profile of previously identified pathogenicity genes. Raw RNA-sequencing reads from two studies (Lowe *et al*. 2014, Sonah *et al*. 2016) were obtained from the Sequence Read Archive at NCBI (Leinonen *et al*. 2011), and mapped to the *L. maculans* JN3 genome (Rouxel *et al*. 2011) using Geneious version 11 software. The TPM (Transcripts Per Kilobase Million) was calculated for the 3´ exon of each gene to minimise bias effects against long transcripts. Highly expressed genes were first identified in the data of Lowe et al. (2014) (SRA accessions SRX456552, SRX456553 and SRX456556) and then confirmed using the data of Sonah et al. (2016) (SRA accessions provided in Supplemental Table 1). Genes were chosen primarily on the basis of expression profile (i.e. those most strongly up-regulated *in planta*); however, genes with closely related paralogs that may have redundancy in function were avoided, as were known or predicted avirulence genes.

Plasmid construction

Constructs were generated to express guide RNAs to target Cas9 to *L. maculans* genes that are up-regulated during infection of *B. napus*. The cloning approach was the same as that

used previously (Idnurm *et al*. 2017). Briefly, for each gene an oligonucleotide encoding a partial guide RNA including the region unique to the targeted gene (Table 1) was synthesized (Sigma-Aldrich, Castle Hill, Australia) and then made double stranded through PCR with primers MAI0309 and MAI0310. This double-stranded fragment was cloned into the XhoI site of plasmid pMAI75 (Idnurm *et al*. 2017) using the NEBuilder DNA assembly cloning kit (New England Biolabs, Ipswich, USA) resulting in a construct to express constitutively a guide RNA between two ribozymes for processing.

Fungal transformation and screening of gene "edited" strains

Plasmid pMAI23 (Idnurm *et al*. 2017) and the guide RNA constructs were transformed into *Agrobacterium tumefaciens* strain EHA105 using electroporation as described previously (Urquhart and Idnurm 2017).

The T-DNA from pMAI23, encoding the Cas9 endonuclease and resistance to the selectable agent G418, was transformed into the wild-type *L. maculans* strain D9 (Marcroft *et al*. 2012) using *Agrobacterium*-mediated transformation as described previously (Gardiner and Howlett 2004, Urquhart and Idnurm 2017). A transformant was selected, confirmed to retain pathogenicity, and then separately transformed with each of the guide RNA constructs with selection on medium containing hygromycin. A number of transformants were selected for each construct and single-spored passaged three times before PCR screening with the appropriate primers for each gene (supplemental table 1). Transformants were screened first on the basis of changes in PCR product size, which would indicate a deletion, and then by restriction digest of the PCR product (enzyme details are in supplemental table 1). The nature of the genetic changes in the mutants obtained were subsequently defined by Sanger

148 sequencing at the Australian Genome Research Facility, primarily to identify those with
149 frame-shift mutations that would result in loss-of-function.
150
151 **Pathogenicity assays**
152
153 The pathogenicity of *L. maculans* strains was assessed by preparing a suspension of
154 pycnidiospores (10^6 spores/ml) in sterilized water and inoculating 20 µl of this solution onto
155 the cotyledons of seven-day old *B. napus* cv. Westar plants that had been wounded using a
156 syringe needle. Disease progression was examined every day over a period of 14 days, when
157 the lesions were photographed and assessed for qualitative differences in disease severity
158 using an established scoring index from 0-9 (Koch *et al*. 1991). To quantify disease severity,
159 the lesion areas were measured using ImageJ. The plant disease experiment was repeated
160 twice.
161
162 **RESULTS**
163
164 **Identification of genes up-regulated during the early stages of blackleg disease were**
165 **selected for gene disruption**
166
167 Two datasets are available that cover gene regulation at different stages of disease
168 progression by *L. maculans* on *B. napus*, and relative to growth *in vitro*. Genes selected for
169 disruption were initially chosen based on up-regulation during the cotyledon stage of disease
170 according to the data of Lowe *et al*. (2014). Re-analysis of the raw RNA sequencing reads of
171 Sonah *et al*. (2016) confirmed that all of these genes are up-regulated during infection. The
172 range of up-regulation was from 3.7 to 20,996 fold (Figure 1). Absolute expression values

obtained from the data of Sonah *et al*. (2016) expressed as TPM values are available in supplemental table 2.

In contrast, analyzing the expression profile of the nine previously identified pathogenicity genes revealed up-regulation in only 4 genes (Figure 1, Table 2). The most highly up-regulated of the genes was *Lmpma1* (17.0-fold), which is still much less dramatic than most of the genes chosen for disruption.

Eleven highly up-regulated genes were disrupted by CRISPR-Cas9 induced mutations

17 *L. maculans* genes were targeted for disruption by gene editing. Despite several rounds of passaging the strains transformed with the guide RNA construct, strains with specific gene mutations were obtained for 11 of the genes. Mutants with the alleles featuring frame shift mutations were selected, as shown in Figure 2. The mutations included both single nucleotide insertions/deletions and longer deletions. One mutant was selected per strain as all of these mutations will prevent correct translation of the protein down-stream of the mutation site and thus will render the protein non-functional.

Of the eleven strains with gene disruptions, two displayed obvious *in vitro* phenotypes. Strains with mutations in genes Lema_T006160.1 and Lema_T082980.1 had reduced radial growth rate on a standard growth medium, V8 juice agar (Figure 3).

Pathogenicity is not lost in any of the 11 gene disruption strains

All 11 strains with mutations retained the ability to infect and cause disease symptoms on *B. napus* cotyledons (Figure 4A). The strains consistently produced lesion score averages (Koch et al. 1991) above 6, indicating pathogenicity (data not shown). This demonstrates that none of these highly up-regulated genes are essential for these stages of pathogenesis when they are expressed. Some or all of these genes may make a quantitative contribution towards pathogenicity. Measuring subtle changes in virulence is difficult given the inherent variability in plant infection assays. To address this, the lesion areas were measured and compared to wild type (Figure 4B). For two strains, a statistically significant ($P < 0.01$) difference compared to wild type was observed, with larger lesions for the mutant in Lema_T120090.1 and smaller lesions for the mutant in Lema_T006160.1. The T006160.1 mutant also has a recuded radial growth rate (figure 3), so this loss of pathogenicity might be attributed to a general reduction in fitness. Regardless of these subtle differences, as the focus of this study was to examine the use of RNA-sequencing data as a source of potential targets for fungicide or RNA interference-based disease controls, our primary phenotype of relevance was on a loss in pathogenicity, which is an obvious prerequisite for a useful target.

Of note, the two mutants with slower growth *in vitro* were still able to cause wild type levels of disease. This observation suggests that vegetative growth defects do not imply that a strain will be unable to cause plant disease.

DISCUSSION

A lack of correlation between gene expression and gene function has been observed in other biological contexts. For example, in *Saccharomyces cerevisiae* it has been noted that gene expression in response to DNA-damaging agents does not correlate with UV sensitivity in

corresponding mutant strains (Birrell *et al*. 2002) and that the fitness of deletion mutants in anaerobic chemostat cultures does not correlate with transcript profiles (Tai *et al*. 2007).

The loss of these 11 *L. maculans* genes, which are up-regulated during disease and in some cases show homology to genes implicated in the pathogenicity of other plant pathogens (including a putative LysM effector (Kombrink and Thomma 2013) and a sugar permease (Wahl *et al*. 2010)), does not impair the fungus from causing disease. This might reflect a fundamental feature of *L. maculans'* biology. Pathogens such as *L. maculans* are locked in an evolutionary state of "warfare" with their hosts. Hosts evolve R-genes which encode for proteins recognizing specific fungal proteins known as Avr proteins (Bent and Mackey 2007). The *Avr* genes are some of the most highly expressed genes during infection (Lowe *et al*. 2014; Sonah *et al*. 2016). The fungus is then faced with the option to either lose or modify the Avr protein. As discussed in the introductory paragraphs, *L. maculans* has been able to do this numerous times to "breakdown" the actions of resistance genes bred into *B. napus* cultivars. The fact that *L. maculans* is able to dispense with a number of proteins, likely involved in disease, while still maintaining the ability to infect its host, might therefore represent an important evolutionary adaptability that partly explains its widespread success around the world.

This study demonstrates the ability to make targeted gene disruptions in *L. maculans* using CRISPR-Cas9 modifications, without using positive selection for disruptants as was used to identify mutations in the *hos1* gene that confers resistance to iprodione fungicide (Idnurm *et al*. 2017). It also demonstrates that genes can be specifically targeted using this technology without causing off-target effects that render the fungus non-pathogenic. One caveat to this method is that of the 17 genes initially targeted only 11 could be readily disrupted, suggesting

247 that the efficiency of the technique in *L. maculans* is likely influenced by the target sequence.
248 Variable efficiency in gene targeting by CRIPSR-Cas9 is an established issue (Yarrington *et*
249 *al*. 2018). An alternative hypothesis is that the other six genes may be required for viability
250 of *L. maculans*, and therefore isolating mutants is not feasible.
251
252 The ability to make targeted mutations in *L. maculans* opens up new possibilities in the study
253 of this organism. However, if reverse genetic approaches are to supplant traditional forward
254 genetic screens, an efficient means to identify candidate essential pathogenicity genes will
255 have to be available. The assumption that genes that are highly up-regulated during disease
256 are most likely to be essential for disease is one that we challenge.
257
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266
267 ***Conflict of interest.*** None declared.
268
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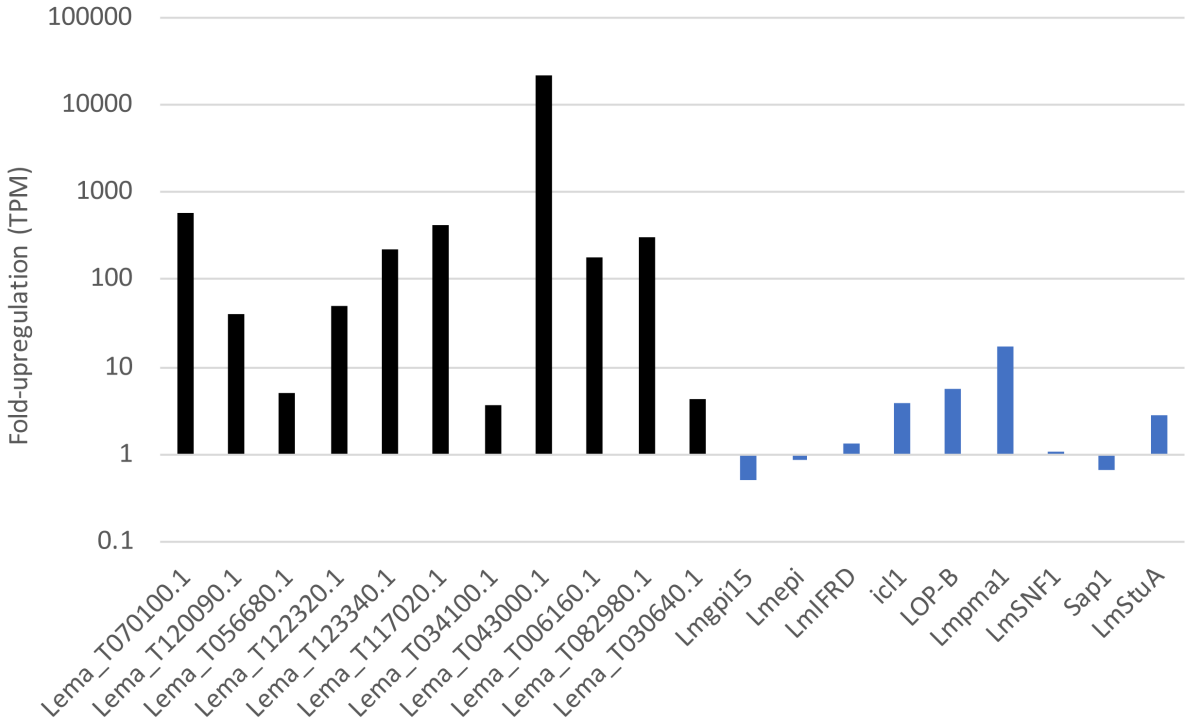
Table 1: Features of the *L. maculans* genes disrupted by CRISPR-Cas9.

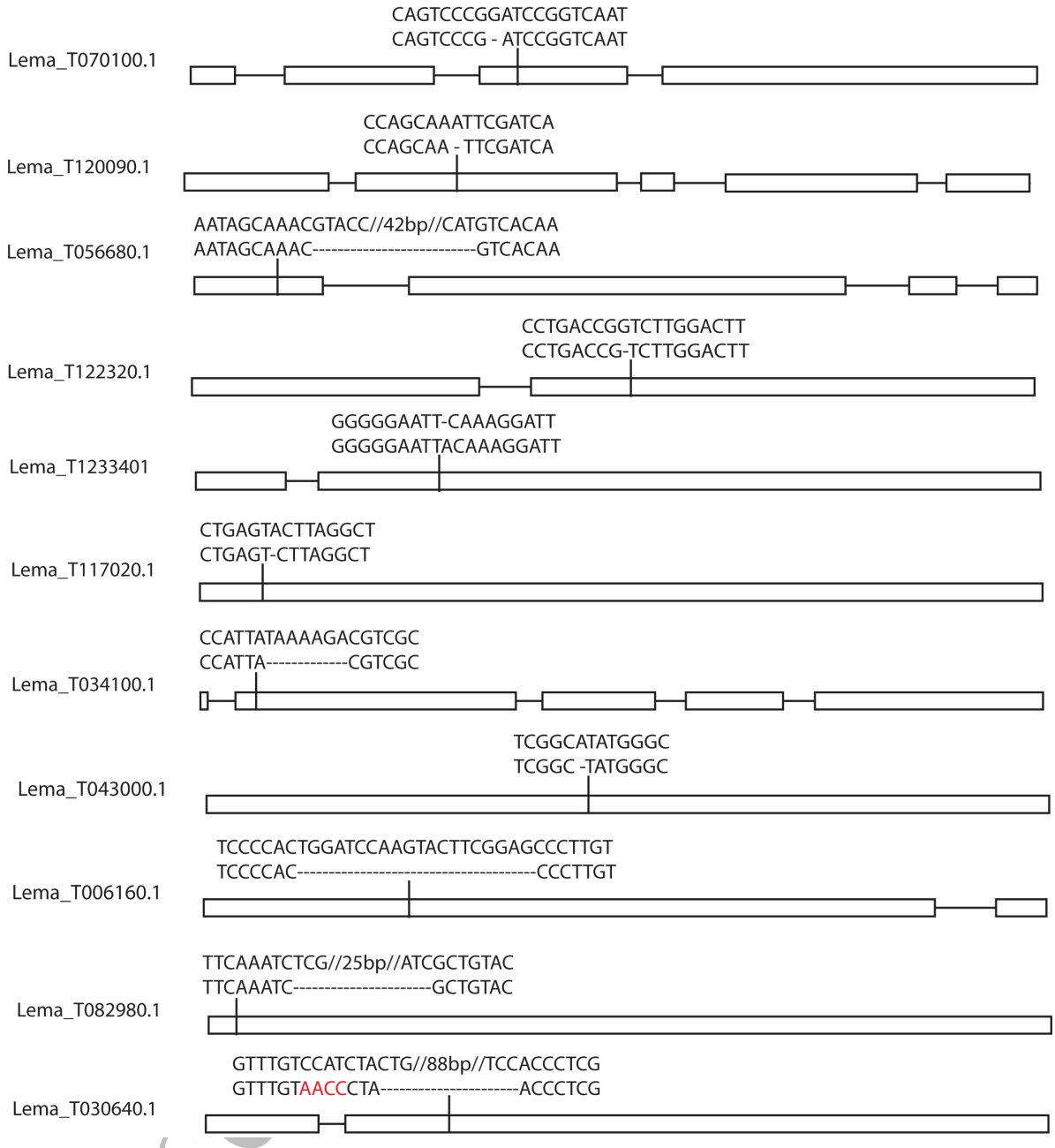
Protein ID	gRNA oligonucleotide [a]	Screening primers [b]	Restriction enzyme for screening [c]	SignalP secretion [d]	Putative function/features [e]
Lema_P070100.1	AU407	F: AU407ScF R: AU407ScR	BamHI	Yes	LysM polysaccharide binding domain
Lema_P120090.1	AU409	F: AU409ScF R: AU409ScR	ApoI	Yes	Monooxygenase
Lema_P056680.1	AU410	F: AU410ScF R: AU410ScR	KpnI	Yes	Glycoside hydrolase family 16 protein
Lema_P122320.1	AU411	F: AU411ScF R: AU411ScR	AgeI	No	Cupin domain
Lema_P123340.1	AU412	F: AU412ScF R: AU412ScR	EcoRI	Yes	Amine oxidase
Lema_P117020.1	AU416	F: AU416ScF R: AU416ScR	ScaI	Yes	Galactosyl transferase
Lema_P034100.1	AU417	F: AU417ScF R: AU417ScR	PsiI	Yes	Polygalacturonase
Lema_P043000.1	AU421	F: AU421ScF R: AU421ScR	NdeI	No	DUF946 domain
Lema_P006160.1	AU424	F: AU424ScF R: AU424ScR	BamHI	Yes	-
Lema_P082980.1	AU425	F: AU425ScF R: AU425ScR	AflIII	Yes	-
Lema_P030640.1	AU426	F: AU426ScF R: AU426ScR	HindIII	No	Fucose/glucose/galactose permease

[a] Primer sequences are provided in supplemental table 1.
[b] Primer sequences are provided in supplemental table 1.
[c] Enzyme used in screening transformants for mutation within the gene target site.
[d] Bioinformatic prediction if the protein encoded by the gene is secreted (Petersen *et al*. 2011).
[e] Functions were inferred from homologs identified by BLAST comparisons.

Table 2: Pathogenicity genes previously identified in *L. maculans*

Gene name	Function	Protein ID	Reference
Lmepi	UDP-glucose-4-epimerase	Lema_P035270.1	(Remy *et al*. 2009)
Lmgpi15	GPI-anchor biosynthesis	Lema_P000130.1	(Remy *et al*. 2008b)
LmIFRD	Required for cell wall integrity	Lema_P000020.1	(Van de Wouw *et al*. 2009)
icl1	Isocitrate lyase	Lema_P000080.1	(Idnurm and Howlett 2002)
LOP-B	Unknown function	Lema_P065010.1	(Idnurm and Howlett 2003)
Lmpma1	Plasma membrane H-ATPase	Lema_P099140.1	(Remy *et al*. 2008a)
LmSNF1	Protein kinase for carbon catabolite-derepression	Lema_P000770.1	(Feng *et al*. 2014)
sap1	Sit4-associated protein	Lema_P071490.1	(Urquhart and Idnurm 2017)
LmStuA	Transcription factor	Lema_P011910.1	(Soyer *et al*. 2015)

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CAGTCCCG - ATCCGGTCAAT
Lema_T070100.1
CCAGCAAATTCGATCA
CCAGCAA - TTCGATCA
Lema_T120090.1
AATAGCAAACGTACC//42bp//CATGTCACAA
AATAGCAAAC----------GTCACAA
Lema_T056680.1
CCTGACCGGTCTTGGACTT
CCTGACCG-TCTTGGACTT
Lema_T122320.1
GGGGGAATT-CAAAGGATT
GGGGGAATTACAAAGGATT
Lema_T1233401
CTGAGTACTTAGGCT
CTGAGT-CTTAGGCT
Lema_T117020.1
CCATTATAAAAGACGTCGC
CCATTA----------CGTCGC
Lema_T034100.1
TCGGCATATGGGC
TCGGC -TATGGGC
Lema_T043000.1
TCCCCACTGGATCCAAGTACTTCGGAGCCCTTGT
TCCCCAC----------CCCTTGT
Lema_T006160.1
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TTCAAATC----------GCTGTAC
Lema_T082980.1
GTTTGTCCATCTACTG//88bp//TCCACCCTCG
GTTTGTAACCCTA----------ACCCTCG
Lema_T030640.1

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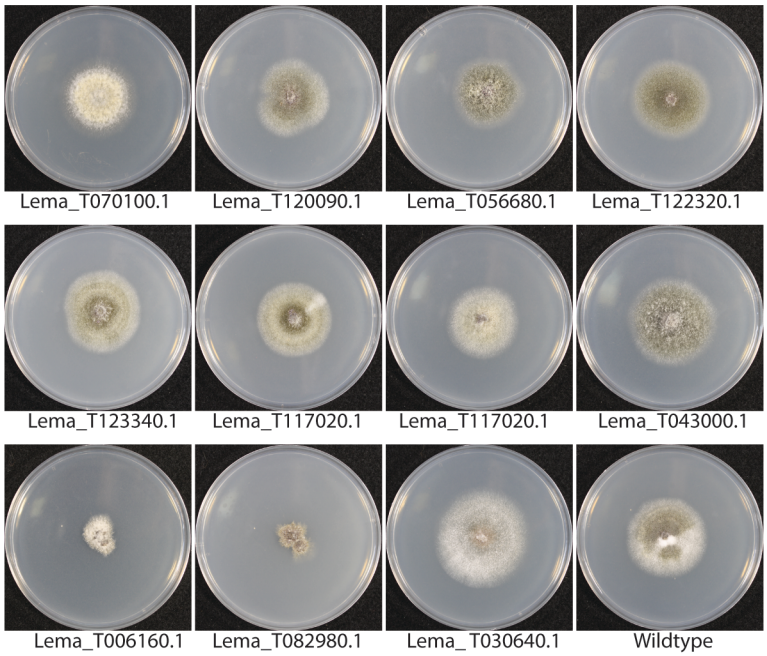

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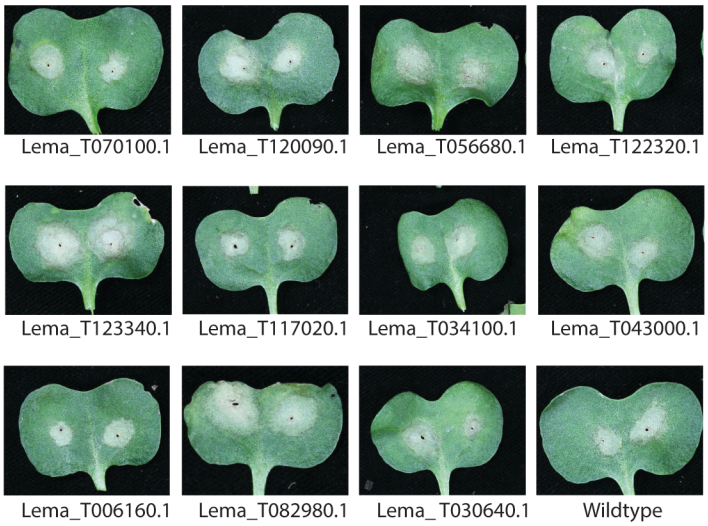
A



B

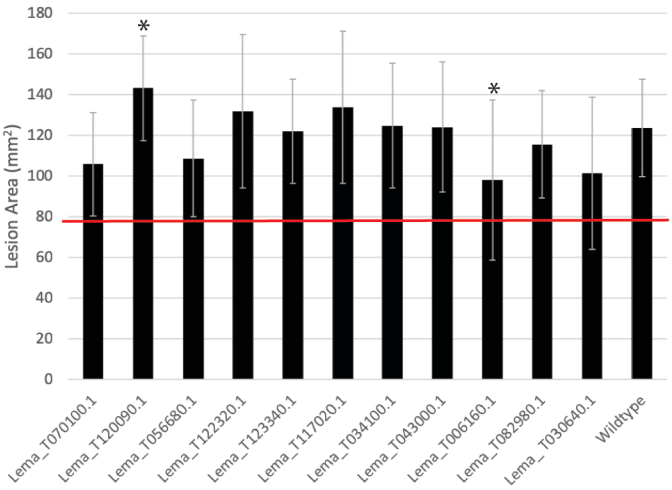


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Figure 1. Up-fold regulation (TPM) of expression of *L. maculans* genes during early infection at seven days post inoculation on *B. napus* compared to *in vitro* culture. The candidates chosen for gene disruption by CRISPR-Cas9 are in black and the previously identified pathogenicity genes are in blue.

Figure 2. Diagrams of the intron-exon structures of the genes targeted for mutation, and the nature of the mutations induced in them using CRISPR-Cas9. Sanger sequencing of potential mutants was conducted to identify those that resulted in reading-frame shift and thus a clear loss of potential function. Sequences of the mutant strains at the site of mutation are displayed below that of the wild type strain.

Figure 3. *In vitro* growth of gene disruptants compared to wild type after seven days on clear V8 juice medium. Two of the mutants showed reduced growth rate (Lema_T006160.1 and Lema_T082980.1). The variation in pigmentation between strains is typical of the parent isolate, D9, in *in vitro* culture.

Figure 4. Deletion of 11 genes with high expression levels during infection does not impair pathogenicity. (A) The strains produced silver-grey lesions similar to the wild type strain D9. (B) Mean lesion areas (from between 17 and 56 lesions) for each strain were calculated. Error bars represent ± 1 standard deviation. Significant differences are indicated with an asterisk (t-test; $P < 0.01$). Red bar indicates the lesion area corresponding to a circular lesion of 1 cm in diameter, representing a highly pathogenic strain.

Supplementary Table 1: Oligonucleotides used in this study

Name	Sequence (5´-3´)
AU407	GAAACCTAATCAATCAACAGTTGTCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCACAACTAACAGTCCCGGATCGTTTTAGAGCTAGAAATAGC
AU407ScF	TATTCAGGTGCCCAAGTGTGC
AU407ScR	CCCAGCAGTAATCCGGCTCG
AU409	GAAACCTAATCAATCAACACAGCCCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCGGCTGTTTGATCGAATTTGCGTTTTAGAGCTAGAAATAGC
AU409ScF	CGACTACATTGAACATACAGC
AU409ScR	CAGTTTTGTGACGTCTTGACC
AU410	GAAACCTAATCAATCAACGCCGTACTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCTACGGCTATCCCTGGGGTACGTTTTAGAGCTAGAAATAGC
AU410ScF	CTGGCGGTACCTACAACAGC
AU410ScR	GCGGTTAGGCGGAGGACACC
AU411	GAAACCTAATCAATCAACGGGAAACTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCTTTCCCATCCTGACCGGTCTGTTTTAGAGCTAGAAATAGC
AU411ScF	CCAGAGACTTCTCACTCAGG
AU411ScR	CAAGTACATAGCCGAAATGG
AU412	GAAACCTAATCAATCAACACTTGCCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCGCAAGTATGGGGGAATTCAAGTTTTAGAGCTAGAAATAGC
AU412ScF	TGAGTTGGGCGGATATGTGG
AU412ScR	ACAGATTACCATAGGTTTGC
AU415	GAAACCTAATCAATCAACATTGCTCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCAGCAATCTCCTTCAAGCTTAGTTTTAGAGCTAGAAATAGC
AU415ScF	TACAAGAGCAACACAGTACGC
AU415ScR	ATACGATCGGTGTGACATCTCC
AU416	GAAACCTAATCAATCAACAAGATTCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCAATCTTGCAACTGAGTACTTGTTTTAGAGCTAGAAATAGC
AU416ScF	CTAGGGATGGGTTATATAGG
AU416ScR	ATTGTTTTGTACCGATAGTGC
AU417	GAAACCTAATCAATCAACGCTGCACTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCTGCAGCGACGTCTTTTATAAGTTTTAGAGCTAGAAATAGC
AU417ScF	CTCATCAGCTTTGCTTGACACC
AU417ScR	CACCCTGAGGCTGCGGTCGTG
AU421	GAAACCTAATCAATCAACTGCTCGCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCCGAGCACAGCGTCGGCATATGTTTTAGAGCTAGAAATAGC
AU421ScF	GGTGACGTCCTTTGGGGAGG
AU421ScR	CTGGTGAGAACGGGAGGTAGG
AU425	GAAACCTAATCAATCAACCGAGATCTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCATCTCGAGAATCAAAACGTGGTTTTAGAGCTAGAAATAGC
AU425ScF	GATTGAAATATTTGCCTTGAAACC
AU425ScR	TTTGACAGAACGTGACATCG
AU426	GAAACCTAATCAATCAACGGCTGACTGATGAGTCCGTGAGGACGAAACGAGTA AGCTCGTCTCAGCCGTCAAACGAAGCTTGTTTTAGAGCTAGAAATAGC
AU426ScF	TCCAAGCTGCCTACTTCGGCGCC
AU426ScR	CGACAGCCTGGACGGACTGG