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Sequence diversity in the large subunit of RNA polymerase I contributes to Mefenoxam insensitivity in *Phytophthora infestans*

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SUMMARY

Phenylamide fungicides have been widely used for the control of oomycete-incited plant diseases for over 30 years. Insensitivity to this chemical class of fungicide was recorded early in its usage history, but the precise protein(s) conditioning insensitivity has proven difficult to determine. To determine the genetic basis of insensitivity and to inform strategies for the cloning of the gene(s) responsible, genetic crosses were established between Mefenoxam sensitive and intermediate insensitive isolates of *Phytophthora infestans*, the potato late blight pathogen. F1 progeny showed the expected semi-dominant phenotypes for Mefenoxam insensitivity and suggested the involvement of multiple loci, complicating the positional cloning of the gene(s) conditioning insensitivity to Mefenoxam. Instead, a candidate gene strategy was used, based on previous observations that the primary effect of phenylamide compounds is to inhibit ribosomal RNA synthesis. The subunits of RNA polymerase I (RNApolI) were sequenced from sensitive and insensitive isolates and F1 progeny. Single nucleotide polymorphisms (SNPs) specific to insensitive field isolates were identified in the gene encoding the large subunit of RNApolI. In a survey of field isolates, SNP T1145A (Y382F) showed an 86% association with Mefenoxam insensitivity. Isolates not showing this association belonged predominantly to one *P. infestans* genotype. The transfer of the 'insensitive' allele of *RPA190* to a sensitive isolate yielded transgenic lines that were insensitive to Mefenoxam. These results demonstrate that sequence variation in RPA190 contributes to insensitivity to Mefenoxam in *P. infestans*.

Keywords: fungicide, Mefenoxam, Metalaxyl, oomycete, phenylamide, *Phytophthora infestans*, RNA polymerase.

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INTRODUCTION

Disease represents one of the most significant sources of crop losses in agriculture. The management of such disease (or the mitigation of such losses) may rely on agronomic practices (crop hygiene), plant resistance or the application of chemicals that are inhibitory to pathogen development. Globally, potato is the third most important food crop, and the most important noncereal crop (Birch *et al*., 2012). The major disease threat to this crop is from *Phytophthora infestans*, causing late blight. *Phytophthora infestans* also infects tomato, which is the ninth most important crop on a global scale (FAOstat, 2011 data; http://faostat.fao.org/). The control of *P. infestans* in potato has been achieved through the deployment of major resistance (*R*) genes. However, the majority of these resistances have been 'broken' by pathogen genotypes that are no longer recognized by the host plant, and are therefore virulent (Fry, 2008). As a consequence, the control of late blight is mostly achieved by the application of inhibitory agrochemicals. Under cool and wet conditions that favour late blight development, chemical application may be required at weekly intervals to maintain disease control (Haverkort *et al*., 2008).

Chemical application has been used to control oomycete diseases for over a century (reviewed in Erwin and Ribeiro, 1996). Early chemicals to control late blight included 'Bordeaux mixture', a broad-spectrum antimicrobial mixture containing copper salts. Subsequent products also had a broad spectrum of activity, such as Mancozeb, which inhibited the growth of fungi and oomycetes (still considered to be fungi at the time). In the past 35 years, chemicals that exhibit specificity to oomycetes have been developed. An early example of these chemicals is the phenylamide Metalaxyl, which is an unresolved isomeric mixture. Subsequently, the (*R*)-enantiomer of Metalaxyl was found to provide the majority of the biological activity against oomycetes. When ≥91% pure, the (*R*)-enantiomer of Metalaxyl (IUPAC name: methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-D-alaninate; methyl (*R*)-2-{[(2,6-dimethylphenyl)methoxyacetyl]amino}propionate) has the common name Metalaxyl-M. Mefenoxam is a Syngenta Trade Mark for a Metalaxyl product which is enriched for the (*R*)-enantiomer (MacBean, 2012). More recently developed oomycete-specific

chemicals include the carboxylic acid amides (Lamberth *et al*., 2008), for which a specific mutation in the oomycete cellulose synthase 3 (*CesA3*) gene has been demonstrated to condition insensitivity in oomycetes (Blum *et al*., 2010a). The mode of action for other chemical control agents that are specific to oomycetes, and target site mutations that lead to insensitivity, are less well characterized (FRAC; http://www.frac.info/index.htm).

Despite the very large amounts of chemical control agents applied to potato crops, there are few reports of naturally occurring chemical insensitivity in *P. infestans*. The most thoroughly documented reports relate to field isolates that are insensitive to phenylamide control chemicals, including Metalaxyl (Davidse *et al*., 1988). This contrasts with the downy mildew, *Plasmopara viticola*, the genotypes of which may show natural insensitivity to other chemical classes (Blum *et al*., 2010b).

Metalaxyl was first used to control oomycete diseases in the late 1970s (Erwin and Ribeiro, 1996). However, pathogen genotypes that were fully insensitive to the chemical were identified soon after its widespread use (reviewed in Gisi and Sierotzki, 2008; Gisi *et al*., 2000). Pathogen strains that were insensitive to Metalaxyl also exhibited cross-resistance to other phenylamide compounds, such as Furalaxyl, Benalaxyl and Oxadixyl (Bruin and Edgington, 1982; Davidse *et al*., 1988; Joseph and Coffey, 1984). The first reports of *P. infestans* field isolates insensitive to Metalaxyl originate from 1980 (Davidse *et al*. 1981). Later testing of *P. infestans* isolates revealed that insensitive genotypes were already present (Dagget *et al*., 1993) before the widespread use of Metalaxyl. Further, it is well documented that phenylamide-insensitive laboratory mutants of *Phytophthora* sp. are readily generated (Bruin and Edgington, 1982; Davidse, 1981; Joseph and Coffey, 1984). Mefenoxam continues to be effective in controlling oomycete diseases in crops (Rebollar-Alviter *et al*. 2010, 2012), and has recently been used to treat infection of animals by *Pythium insidiosum* (Hummel *et al*., 2011).

Genetic studies to determine the nature of insensitivity to Metalaxyl have involved crosses between insensitive and sensitive isolates of various *Phytophthora* sp. Segregation patterns of the trait among progeny revealed a range of insensitivity levels, with many intermediate between parental isolates (reviewed in Gisi and Sierotzki, 2008; Gisi *et al*., 2000). Insensitivity is proposed to be conditioned by one or sometimes two incompletely dominant loci, called *MEX1* and *MEX2* (Judelson and Roberts, 1999; Lee *et al*., 1999). Additional minor genes may also contribute to the level of insensitivity (Fabritius *et al*., 1997; Judelson and Senthil, 2006; Lee *et al*., 1999).

Biochemical assays using [^{3}H]uridine incorporation revealed a specific impact of Metalaxyl on the synthesis of RNA (Davidse *et al*., 1983, 1988) and, specifically, ribosomal RNA (rRNA) (Davidse *et al*., 1983; Wollgiehn *et al*., 1984). The syntheses of messenger and transfer RNAs (mRNA and tRNA, respectively) were affected to a lesser extent. This implicated RNA polymerase I (RNApolI), as it transcribes rRNA. Furthermore, this study also suggested that Metalaxyl exerted its activity when the RNA polymerase complex was bound to DNA (Davidse *et al*., 1983). Eukaryotic DNA-dependent RNA polymerases are multi-subunit complexes, and up to seven subunits may be shared among the three major RNA polymerases (Kuhn *et al*., 2007; Schneider, 2012). Additional proteins, such as topoisomerases and transcription factors, may also act to influence RNA polymerase activity (Drygin *et al*., 2010). These factors have complicated the identification of the precise RNA polymerase subunit targeted by Mefenoxam, and the sequence variability that leads to insensitivity.

The genome sequences for several oomycete species (reviewed in Raffaele and Kamoun, 2012) will help to link genetic and genomic resources to determine the precise target for Mefenoxam. A genetic map has been developed for *P. infestans* (van der Lee *et al*., 1997, 2004), together with a large insert bacterial artificial chromosome library (Whisson *et al*., 2001), and a genome sequence (Haas *et al*., 2009). Transformation of *P. infestans* is also possible (Judelson *et al*., 1991). All of these resources can be combined in a positional (map-based) cloning strategy to isolate the gene(s) conditioning Mefenoxam insensitivity.

Here, we have established a genetic cross between sensitive and intermediate insensitive isolates of *P. infestans*. The recovered progeny displayed different insensitivity rankings at varying concentrations of Mefenoxam, with segregation suggesting involvement of more than a single locus. This, combined with the small progeny population, led us to use a candidate gene strategy as an alternative approach. The identification and sequencing of genes encoding RNApolI subunits from the genome sequence of *P. infestans* revealed numerous single nucleotide polymorphisms (SNPs). A small number of SNPs in the gene encoding the large subunit of RNApolI were specific to insensitive isolates amongst a panel of *P. infestans* isolates of known sensitivity. The transfer of this allele into a sensitive isolate yielded insensitive lines, confirming that this gene contributes to Mefenoxam insensitivity in *P. infestans*.

RESULTS

Genetics of Mefenoxam insensitivity in *P. infestans*

A cross was established between isolates 88133 (A2 mating type; sensitive) and T30-4 (A1 mating type; intermediate insensitive). T30-4 is an F1 derived from a cross between 88133 and 80029 (van der Lee *et al*., 1997; Whisson *et al*., 2001). Isolate 80029 is insensitive (Fig. 1), and is assumed to be *MEX/MEX*, whereas isolate 88133 is fully sensitive, and is assumed to be *mex/mex*. Thus, it was assumed that T30-4 was *MEX/mex* and that the F1 progeny from the 88133 × T30-4 cross would segregate in a 1:1

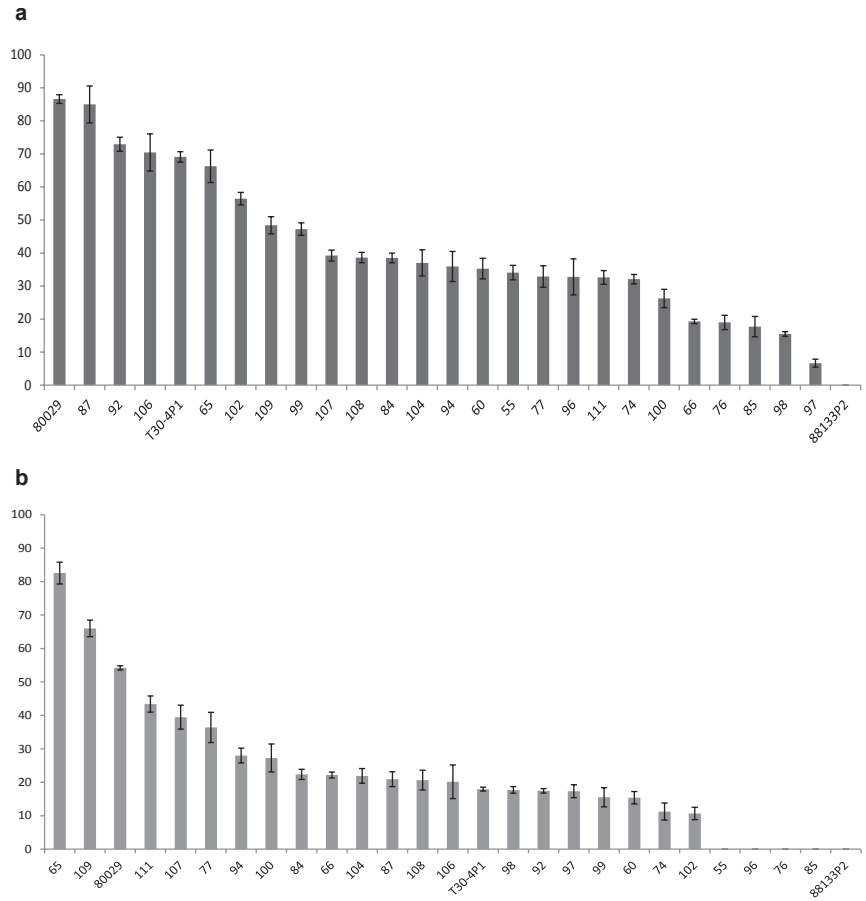

Fig. 1 Growth of *Phytophthora infestans* F1 progeny from a cross of 88133 × T30-4 on rye agar amended with 5 μg/mL (a) and 100 μg/mL (b) Mefenoxam. Growth on Mefenoxam-amended medium is presented as a percentage of growth of the culture on medium containing no Mefenoxam (y axis). Parental strains and F1 progeny are shown on the x axis. Parental isolate 88133 is fully sensitive to Mefenoxam, whereas the T30-4 parental isolate is intermediate insensitive. Isolate 80029, a parent of T30-4, is included here for comparison. Data are the mean of 12 measurements; standard deviation from the mean is shown for each *P. infestans* line.

ratio for this trait. Less than 1% of oospores germinated on water agar medium over a period of 30 days; 450 germinating oospores were individually transferred to rye agar without any selective antibiotic, and 150 continued to grow into colonies. Screening using a total of 31 amplified fragment length polymorphism (AFLP) markers (van der Lee *et al*., 1997) revealed that only 24 cultures had inherited markers from both parental isolates, and thus were genuine F1 progeny. Only a single self-derived progeny from T30-4 was identified. No self-derived progeny from 88133 were identified. The self-derived culture of T30-4 was not retained in further analyses as only a single progeny of this type would not prove informative.

The parental isolates and F1 progeny were tested for growth on rye agar containing 5 and 100 μg/mL Mefenoxam. At the lowest dose, growth of the parental isolate 88133 was completely inhibited, whereas T30-4 showed an intermediate level of inhibition (31%). Isolate 80029, a parent of T30-4, was included for comparison. At both 5 and 100 μg/mL Mefenoxam, this isolate exhibited less growth inhibition than T30-4, consistent with it being homozygous for the Mefenoxam insensitivity trait, and T30-4 being heterozygous. In contrast with parental isolate 88133, none of the F1 progeny were fully inhibited by 5 μg/mL Mefenoxam and, instead, showed a range of sensitivity, from 15% to 93% growth inhibition (Fig. 1a). Two F1 progeny (F1-87, F1-92) exhibited a significantly higher level of insensitivity than the intermediate T30-4 parent.

At 100 μg/mL Mefenoxam, the parental isolate T30-4 showed 80% growth inhibition. In contrast, at this Mefenoxam concentration, 11 of the F1 progeny exhibited a significantly higher level of insensitivity than the T30-4 parent (Fig. 1b). The most insensitive progeny, F1-65 and F1-109, exhibited 17% and 34% growth inhibition, respectively. Four F1 progeny, F1-55, F1-76, F1-85 and F1-96, were fully sensitive to 100 μg/mL Mefenoxam.

Comparing the two sets of results, the ranking of F1 progeny for their insensitivity was re-assorted at each dosage. As an example, progeny F1-55 and F1-96, which were intermediate insensitive at the 5 μg/mL dosage, were fully sensitive at 100 μg/mL. F1-97 and F1-98, the most sensitive progeny at 5 μg/mL, were not significantly different from the T30-4 parent at 100 μg/mL. This re-assortment of F1 progeny insensitivity rankings at the different doses tested, combined with the 20:4 (insensitive : sensitive) segregation, is suggestive of more than a single locus contributing to the insensitive phenotype in the cross described here. The apparent genetic complexity, combined with the small F1 population size, signified that it would be problematic to continue with positional (map-based) cloning of the locus conditioning insensitivity.

RNApolI in *P. infestans*

The core of RNApolI is typically a 14-subunit protein complex in eukaryotes (Kuhn *et al*., 2007). From the *P. infestans* genome sequence, genes encoding 12 RNApolI subunits were identified (Table 1). The gene encoding the RNApolI-specific factor RRN3 was also identified. Although expressed sequence tags (ESTs) encoding subunits RPABC10α and RPABC10β had been identified in an earlier study (Randall *et al*., 2005), these were not annotated as genes in the *P. infestans* genome. RPABC10α and RPABC10β were identified as single copy genes in *P. infestans* by TBLASTN searches using orthologous sequences from yeast. Genes encoding subunits RPA14 and RPA34.5 were not found in the *P. infestans* genome. The genes encoding these subunits were also not identified in the sequenced genomes of *P. sojae* and *P. ramorum* (Tyler *et al*., 2006), *P. capsici* (Lamour *et al*., 2012a), *Hyaloperonospora arabidopsidis* (Baxter *et al*., 2010) or *Pythium ultimum* (Lévesque *et al*. 2010), suggesting that the oomycete evolutionary lineage has fewer RNApolI subunits than other well-studied organisms, such as humans and yeasts (Drygin *et al*., 2010; Kuhn *et al*., 2007).

SNPs in RNApolI subunits

Assuming that sequence differences would exist between sensitive and insensitive isolates, the identified RNApolI subunits were initially sequenced from isolates 80029, 88133 and T30-4. Isolate 88133 (sensitive) would be expected to be homozygous at all loci connected with the Mefenoxam phenotype. Candidate SNPs that associate with insensitivity may therefore be expected to be homozygous or heterozygous in 80029 or T30-4. No sequence information was obtained from *RPAC40* (PITG_16658 copy) as this sequence failed to amplify with primer combinations that should have been specific to this gene paralogue. No SNPs predicted to encode amino acid substitutions were found in *RPABC10α*, *RPABC10β*, *RPA43* or *RRN3*, and these genes were excluded from further analyses. SNPs were found in *RPAC40* (PITG_18777 copy), *RPAC19*, *RPABC27*, *RPABC23*, *RPABC14.5*, *RPA49* and *RPA12.2*, but were heterozygous in 88133 (sensitive) or 80029 (insensitive) only, whereas T30-4 carried the allele found in the sensitive isolate. These genes were also eliminated as candidates for conditioning Mefenoxam insensitivity. SNPs present in 80029 and T30-4, but not in 88133, and leading to predicted changes in the amino acid sequence of the encoded proteins, were identified for *RPA190*, *RPAC40* (PITG_16659 copy) and *RPA135* (summarized in Tables S1 and S2, see Supporting Information).

To investigate whether one or more of these subunits may condition insensitivity to Mefenoxam, specific SNPs (*RPA190* T1145A; *RPA135* C1251A; *RPAC40* A867T) were sequenced in the 24 F1 progeny from the 88133 × T30-4 cross, described earlier. No segregation of the tested SNP was observed among the F1 progeny for *RPA135* (Table S3, see Supporting Information). Thus, this subunit was not considered to be a strong candidate for conditioning insensitivity to Mefenoxam.

In contrast, SNPs in *RPAC40* (PITG_16659 copy) and *RPA190* did segregate among the F1 progeny, but neither showed co-segregation with the insensitivity phenotype (Table S3). The *RPAC40* subunit is in common with the RNApolIII complex that transcribes tRNAs. Strong changes in tRNA abundance were not the major effect of Metalaxyl (Wollgiehn *et al*., 1984), and it is thus unlikely that SNPs in this subunit contribute significantly to conditioning insensitivity.

The entire *RPA190* gene was sequenced from nine F1 progeny (four sensitive and five insensitive): F1-55, F1-65, F1-76, F1-77, F1-85, F1-96, F1-107, F1-109, F1-111. This revealed evidence for mitotic gene conversion events (loss of heterozygosity) in F1-77, F1-96, F1-109 and F1-111. This was evidenced by a pattern of homozygous and heterozygous SNPs in the *RPA190* gene in indi-

Table 1 Genes encoding the subunits of RNA polymerase I (RNApolI) in *Phytophthora infestans*.

Polymerase complex	Subunit	*P. infestans* gene	Subunit length (amino acids)	RNApolI subunit specificity
Core	RPA190	PITG_03855	1812	PolI
	RPA135	PITG_02420	1180	PolI
	RPAC40	PITG_16659	340	PolI, III
		PITG_16658	305	
		PITG_18777	344	
	RPAC19	PITG_05854	108	PolI, III
	RPA12.2	PITG_06706	126	PolI
	RPABC27 (RPB5)	PITG_10445	207	PolI, II, III
	RPABC23 (RPB6)	PITG_12877	137	PolI, II, III
	RPABC14.5 (RPB8)	PITG_09712	143	PolI, II, III
	RPABC10β (RPB10)	Supercontig1.27 1022726–1022969	80	PolI, II, III
	RPABC10α (RPB12)	Supercontig1.1 297200–297548	107	PolI, II, III
Subcomplex A14/43	RPA14	Not found	–	PolI
	RPA43	PITG_14613	232	PolI
Subcomplex A49/34.5	RPA49	PITG_11365	430	PolI
	RPA34.5	Not found	–	PolI
Specific transcription factor	RRN3	PITG_15566	788	PolI

vidual F1 progeny. Loss of heterozygosity across *RPA190* appeared not to be randomly distributed, occurring at similar points in the gene for F1-77, F1-96 and F1-111: 1938–2193 and 2611–2989 bp (Table S4, see Supporting Information). In F1-109, loss of heterozygosity occurred between 2436–2553 and 3345–3477 bp.

Location of SNPs in the large subunit (*RPA190*) of RNApoll in *P. infestans*

Although genetics did not identify co-segregation of the T1145A SNP in *RPA190* with Mefenoxam insensitivity, the large number of SNPs in this gene, combined with the loss of heterozygosity events, suggested that further analysis of this gene in field isolates may be warranted. A total of 45 SNPs were identified in *RPA190* between isolates 80029 and 88133. Of these, three were found to be heterozygous in the sensitive isolate 88133 and two were found to be heterozygous in the insensitive isolate 80029. The remaining 40 SNPs between these isolates were homozygous in 80029 and 88133, and were heterozygous in T30-4 (an F1 from 80029 × 88133). Of the 45 SNPs identified, only nine were predicted to encode changes in the amino acid sequence of the predicted protein. Three substitutions were located in the clamp domain, one in the active site, four in the cleft domain and one not associated with functional domains (Fig. 2, Table S2). No sequence information could be determined from the first 610 bp of the *RPA190* gene, as repeated attempts to sequence this region in forward or reverse directions were unsuccessful.

The *RPA190* gene was sequenced from an additional 12 *P. infestans* isolates to identify additional SNPs and to determine whether there was any evidence for SNP association with Mefenoxam insensitivity at a field isolate level. The additional isolates originated from different geographical regions (USA, Mexico, UK, Europe) and different years (1980–2007). This increased the total number of polymorphic nucleotide sites identified in the *RPA190* gene to 74, 20 of which encode amino acid substitutions in the predicted protein. The distribution of all substitutions showed that six were located in the clamp domain, three in the active site, one in the funnel domain, eight in the cleft domain and two not associated with functional domains (Fig. 2, Table S2).

Association of *RPA190* SNPs with insensitivity to Mefenoxam

RPA190 sequences were grouped by the chemical sensitivity phenotype of the isolates, and SNPs were sought that occurred only in the insensitive isolates. It is possible that insensitivity to Mefenoxam may have arisen on multiple occasions in different pathogen genotypes, potentially leading to independent SNPs encoding changes that lead to insensitivity. Thus, a specific SNP would not necessarily occur in all insensitive isolates. With this consideration, up to 13 SNPs in *RPA190* have the potential to contribute to conditioning insensitivity to Mefenoxam (Fig. 2; Table S5, see Supporting Information). Of these, one SNP associated fully with the chemical sensitivity phenotype among the 15 isolates. This SNP encodes a tyrosine–phenylalanine substitution at position 382 (sensitive T1145A insensitive) in the clamp domain. Other SNPs that may condition insensitivity also showed an association with background genotype, such as (for example) isolates 80029, 88069, T30-4 and 4910, which are European or UK isolates that have a very similar SNP profile for *RPA190*, despite originating from years 1980–2007. Similarly, insensitive isolates 06_3928A and 07_5314C, belonging to the prevalent '13_A2' UK genotype (Cooke *et al*., 2012), exhibited SNPs that were specific to that genotype.

As the T1145A SNP showed an association with the Mefenoxam sensitivity phenotype in 15 isolates, this region of *RPA190* was sequenced from an additional 22 isolates. In this extended set of isolates, five showed a mismatch between the T1145A SNP and Mefenoxam sensitivity phenotype. One of these five mismatched isolates was background genotype UK ML-SSR genotype 8 (Cooke *et al*., 2012), and was scored as insensitive, whilst exhibiting a homozygous T1145 SNP genotype. A further three isolates of the same background genotype exhibited a homozygous T1145 SNP genotype, but were scored as intermediate sensitivity (Table 2). The remaining isolate, of a different back-

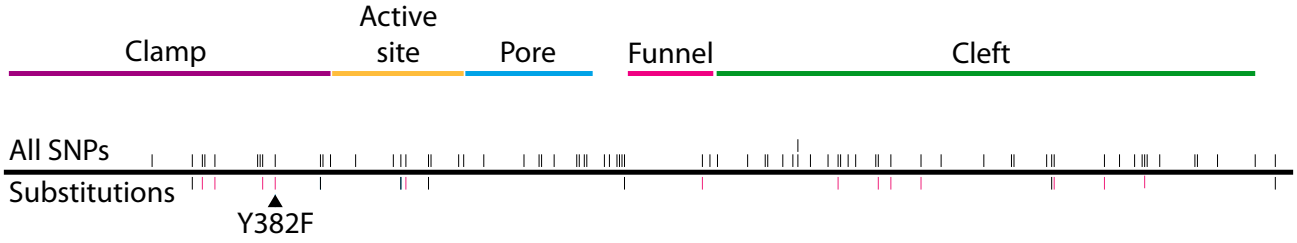

Fig. 2 Organization of predicted functional domains within *Phytophthora infestans* RPA190, showing the location of single nucleotide polymorphisms (SNPs) identified from 15 isolates (shown to scale). The *RPA190* gene sequence is shown as a solid black line. Regions encoding the functional domains of RPA190 are shown above the gene sequence: clamp (purple), active site (yellow), pore (blue), funnel (pink) and cleft (green). SNPs identified from 15 *P. infestans* isolates are shown as short vertical black bars above the solid black line of the gene; a double vertical bar represents two SNPs within a single codon. SNPs leading to changes in the amino acid sequence of RPA190 are shown under the solid black line of the gene; pink bars represent amino acid substitutions found only in isolates that are insensitive to Mefenoxam. T1145A (Y382F) is indicated by a black triangle.

Table 2 Association of the RPA190 Y382F substitution with sensitivity to Mefenoxam in *Phytophthora infestans* isolates.

Isolate	Country of origin, year	Mefenoxam sensitivity†	*RPA190* 1145 SNP genotype
80029	Netherlands, 1980	R	A
88069	Netherlands, 1988	R	A
T30-4	Netherlands (F1 of 80029 × 88133)	I	A/T
07_4910A	UK, 2007	R	A/T
96_9_5_1	UK, 1996	R	A/T
95_1_1_2	UK, 1995	R	A/T
07_4818A	UK, 2007	I	A/T
06_3928A	UK, 2006	R	A/T
07_5314C	UK, 2007	R	A/T
88133	Netherlands, 1988	S	T
95_8_3_1	UK, 1995	S	T
95_8_4_2	UK, 1995	S	T
Ca65	USA, 1980	S	T
550	Mexico, 1983	S	T
PIC99189	Mexico 1999	S	T
96_17_5_3	UK, 1996	S	T
95_17_3_2	UK, 1995	S	T
96_13_1_3	UK, 1996	R	A/T
US_467	USA, 1998	R	A/T
EC1	Ecuador, 1998	S	T
AR4	Argentina, 1997	R	A/T
97_28_1_2	UK, 1997	I	A/T
03_7_1_1	UK, 2003	S	T
03_3_2_2	UK, 2003	R	A/T
03_36_4_1	UK, 2003	R	A/T
03_33_1_1	UK, 2003	S	T
03_16_3_1	UK, 2003	R	A/T
03_31_1_2	UK, 2003	R	A/T
03_32_1_2	UK, 2003	R	A/T
06_3880A	UK, 2006	S	T
06_3884A	UK, 2006	R	A/T
06_3888A	UK, 2006	R	A/T
03_5_3_1	UK, 2003	S	A/T
95_16_3_1	UK, 1995	R*	T
97_38_2_2	UK, 1997	I*	T
03_17_3_3	UK, 2003	I*	T
03_28_1_2	UK, 2003	I*	T

SNP, single nucleotide polymorphism.

*These isolates all belong to a single background genotype.

†S, sensitive; I, intermediate insensitive; R, insensitive/resistant.

ground genotype, was scored as sensitive, but was heterozygous T1145A. In total, the T1145A SNP exhibited an 86% (32/37) association with the Mefenoxam sensitivity phenotype (Table 2).

Transformation of Mefenoxam-sensitive *P. infestans* with alleles of *RPA190*

To investigate whether sequence variation in *RPA190* contributes to Mefenoxam insensitivity, two different alleles, each representing the entire 5.4-kb *RPA190* gene, were cloned into the plasmid vector pTor for constitutive expression in *P. infestans*. One allele was cloned from fully sensitive isolate 88133, and one allele from insensitive isolate 80029. In confirming that the correct sequences had been cloned, it was also revealed that there were no additional SNPs present in the first 610 bp of *RPA190* for isolates 80029 and 88133.

Both alleles were transformed, separately, into sensitive isolate Ca65. Four lines that grew on geneticin selection were recovered from transformations using the 88133 allele (sensitive allele), and nine lines from transformations using the 80029 allele (insensitive allele). The nontransformed Ca65 isolate and all transgenic lines were assessed for growth on rye agar amended with 5, 10, 25, 50 and 100 μg/mL Mefenoxam. The nontransformed Ca65 isolate did not grow at any of the Mefenoxam concentrations. Similarly, none of the four lines transformed with the sensitive allele grew at any Mefenoxam concentration. In contrast, three of the nine lines (1-4, 17-5, 60-2) transformed with the insensitive allele continued to grow at the highest Mefenoxam concentration (Fig. 3, Table 3). Line 17-5 exhibited only limited growth on medium containing Mefenoxam. However, lines 60-2 and 1-4 were not greatly inhibited by the presence of the chemical, when compared with their growth in the absence of Mefenoxam. Reverse transcription-

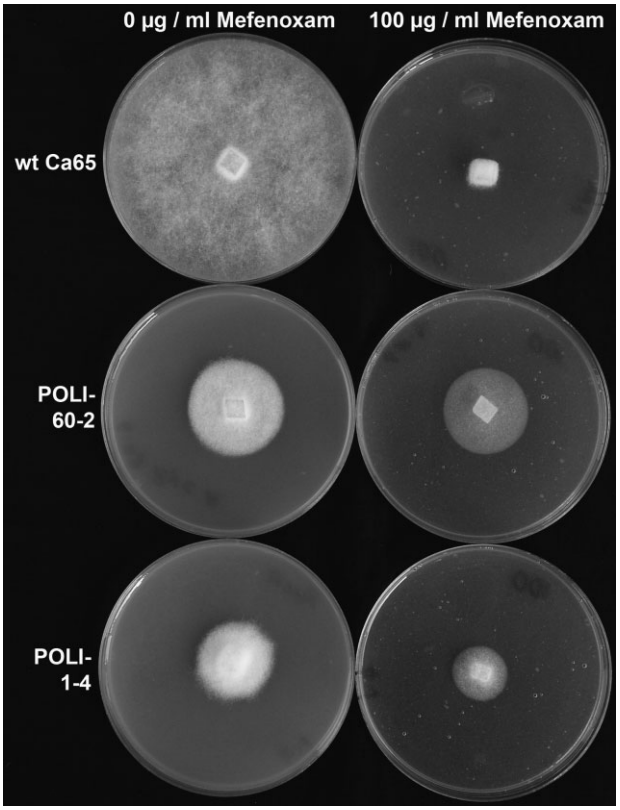

Fig. 3 Transfer of the *RPA190* allele from an insensitive isolate of *Phytophthora infestans* to sensitive isolate Ca65. Wild-type Ca65 (top) exhibits only minimal aerial growth at a high dose (100 µg/mL). Transgenic lines POLI-60-2 and POLI-1–4 grow slowly in the absence of Mefenoxam, but continue to grow into the growth medium at 100 µg/mL of Mefenoxam. Image was taken after 12 days of growth in 90-mm-diameter Petri dishes.

polymerase chain reaction (RT-PCR) was used to confirm that the transgenic lines expressed the introduced allele (Table 3). It was notable that lines expressing introduced alleles of *RPA190* grew more slowly in the absence of Mefenoxam or other antibiotic (compared with nontransformed Ca65) (Fig. 3), although some lines not expressing the introduced allele of *RPA190* also grew more slowly than Ca65 (Fig. S1, see Supporting Information). The allele-specific effects in the transgenic lines indicate that sequence variation in *RPA190* can contribute to insensitivity to Mefenoxam.

DISCUSSION

The specific proteins that condition insensitivity, and thus mode of action, to the oomycete-specific control chemical Metalaxyl/Mefenoxam have been sought for over three decades. Here, we have identified variation in the large subunit of RNApolI (RPA190) as contributing to insensitivity to this chemical in *P. infestans*. However, it is likely that additional factors that contribute to this phenotype remain to be identified.

Previous genetic studies have suggested that insensitivity to Metalaxyl is conditioned by one or two major *MEX* loci with minor effects contributed by other genes (Fabritius *et al*., 1997; Judelson and Roberts, 1999; Judelson and Senthil, 2006; Knapova *et al*., 2002; Lee *et al*., 1999). Here, we also identified a profile of inheritance that suggested the involvement of more than one locus. However, greater genetic dissection was hampered by the small numbers of F1 progeny recovered from our *P. infestans* cross, and differing phenotypic rankings of the F1 progeny at low and high Mefenoxam concentrations. Minor loci contributing to chemical insensitivity may include nonspecific efflux pumps and detoxification. These functions may be carried out by proteins such as ATP binding cassette (ABC) transporters and cytochrome P450 proteins, respectively (Duffy *et al*., 2003; Yoo and Lee, 2011). However, *P. infestans* isolates that have intrinsically elevated levels of nonspecific insensitivity to chemicals do not show corresponding elevation in transcript abundance from genes encoding ABC transporters (Judelson and Senthil, 2006). In the study by Judelson and Senthil (2006), nonspecific mechanisms that contribute to variation in chemical sensitivity were shown to be heritable. It is possible that nonspecific mechanisms contributing to insensitivity may have influenced the ranking of the F1 progeny in the cross described in the present study.

Bulked segregant analysis to identify DNA markers linked to *MEX* was not considered to be feasible in the present study because of the combination of low progeny numbers (especially sensitive progeny) and variable responses to differing concentrations of Mefenoxam. Previous studies examining *MEX* inheritance have also used relatively small numbers of F1 progeny (Judelson and Roberts, 1999). Despite the small progeny size, genetically linked random amplified polymorphic DNA (RAPD) markers were identified with a recombination fraction of 0.11 (Fabritius *et al*., 1997; Judelson and Roberts, 1999). Extension of one cross to over 200 progeny confirmed the linkage of the RAPD markers (Judelson and Senthil, 2006), although the association was not absolute. The genetic distances of these markers from the *MEX* locus would probably have precluded their use in the current study.

To avoid the influence of minor genes, and to circumvent the limitations of a small F1 population size, we exploited the existing evidence for the effects of Metalaxyl on oomycetes (Davidse *et al*., 1983, 1988; Wollgiehn *et al*., 1984), and the availability of the *P. infestans* genome sequence (Haas *et al*., 2009). Previously, a similar combination of biochemical evidence and phenotype, and candidate gene strategy, was used to identify the mode of action for the oomycete control chemical Mandipropamid (Blum *et al*., 2010a). The *P. infestans* avirulence gene *PiAvr3a* was also identified by the association of phenotype with SNPs in a candidate gene in field isolates (Armstrong *et al*., 2005). RNApolI transcribes rRNA in eukaryotes and, based on biochemical evidence, this complex of subunits is the most likely target for Mefenoxam. Prior to the genome sequencing of *P. infestans*, previous EST sequenc-

Table 3 Growth of *Phytophthora infestans* transgenic lines expressing 'insensitive' or 'sensitive' alleles of RPA190 on medium amended with 5 and 100 μg/mL Mefenoxam.

Culture	Growth on Mefenoxam	Transgenic *RPA190* expression	Comments
Ca65	–	–	Sensitive wild-type
P. infestans Ca65 transformed with insensitive allele of *RPA190*			
PolI-1-3	–	–	
PolI-1-4	+	+	
PolI-60-2	+	+	
PolI-60-3	–	–	
PolI-60-4	–	–	
PolI-60-5	–	–	
PolI-60-7	–	–	
PolI-17-1	–	–	
Pol1-17-5	+/–	+	Growth only at 5 μg/mL Mefenoxam
P. infestans Ca65 transformed with sensitive allele of *RPA190*			
PolI-sens1	–	–	
PolI-sens3	–	–	
PolI-sens4	–	+	
PolI-sens5	–	–	

ing projects (Kamoun *et al*., 1999; Randall *et al*., 2005) did not yield full sequence coverage for the largest subunits of this enzyme complex, or indeed represent the full range of subunits. In other eukaryotes, RNApolI is composed of 14 subunits. However, genes encoding only 12 of the 14 subunits could be identified from the genome sequences of *P. infestans* or other sequenced oomycete genomes, suggesting that the oomycetes have a simpler RNApolI organization. The subunits that were not identified, RPA14 and RPA34, have been reported to act in separate dimer subcomplexes (Kuhn *et al*., 2007). In the yeast *Saccharomyces cerevisiae*, these two proteins are individually not essential for RNApolI activity (Beckouet *et al*., 2008; Smid *et al*., 1995).

From the sequencing of the genes encoding the RNApolI subunits and the associated RRN3 transcription factor in parental isolates 80029 (presumed to be homozygous *MEX/MEX* or *MEX/mex*), T30-4 (presumed to be *MEX/mex*) and 88133 (homozygous *mex/mex*), all but three subunits could be eliminated from further analysis. Although SNPs were identified, *RPAC40* was considered less likely to be involved in conditioning insensitivity to Mefenoxam, as it is shared with RNApolIII, and Metalaxyl treatment led to a stronger reduction in rRNA than tRNA levels (Wollgiehn *et al*., 1984). SNPs from *RPA190*, *RPA135* and *RPAC40* in F1 progeny did not co-segregate with the Mefenoxam insensitivity phenotype. As a result of the large number of SNPs identified, and evidence for loss of heterozygosity events, we investigated *RPA190* further in diverse field isolates to determine whether any of the identified SNPs would exhibit an association with Mefenoxam insensitivity. This strategy would also address the possibility that the locus (or loci) conditioning Mefenoxam insensitivity in the cross described here may differ from that in other field isolates.

As a large number of SNPs were identified that led to coding changes in RPA190, it was difficult to definitively associate any particular SNP with insensitivity. Sequencing from 15 isolates identified a single SNP that associated completely with insensitivity, but this association was not absolute (86%) when additional isolates were tested. Interestingly, four of the five mismatched isolates were UK isolates of background RF006 RG57 fingerprint (UK ML-SSR genotype 8; Cooke *et al*., 2012). This genotype encompasses diverse isolates with variable levels of Mefenoxam insensitivity (Day *et al*., 2004). Either different SNPs in *RPA190* lead to Mefenoxam insensitivity in this genotype, or a different locus contributes to the phenotype. The first of these explanations implies that Mefenoxam insensitivity has arisen on independent occasions through sequence variation in *RPA190*; insensitivity occurs in multiple clonal genotypes of *P. infestans* from different geographical locations (Table 2). Alternatively, genetic studies have suggested that a second locus (*MEX2*), distantly linked to *MEX1* (recombination fraction > 0.6), contributes to Metalaxyl insensitivity in some isolates (Fabritius *et al*., 1997; Judelson and Roberts, 1999). From the phenotypic assay of F1 progeny from the cross described here, it is possible that two or more genes conditioning Mefenoxam insensitivity were involved. If either gene can condition insensitivity, and one does not segregate in a cross encompassing both loci (for example *RPA135* in the present cross), additional contributing loci (for example *RPA190*) could segregate independently, potentially without influencing the phenotype. This model may explain why the tested SNPs did not co-segregate with the insensitivity phenotype in the cross, whereas the survey of field isolates revealed an 86% association for the T1145A SNP, and allele transfer experiments showed RPA190 involvement in insensitivity. RNApolI is a multi-subunit enzyme, and therefore it is possible that sequence variation in other subunits may also lead to Mefenoxam insensitivity. In the wheat fungal pathogen *Mycosphaerella graminicola*, induced insensitivity to the succinate dehydrogenase inhibitor (SDHI) fun-

gicides may be conditioned by sequence variation in multiple subunits of the enzyme complex (Scalliet *et al*., 2012).

The analysis of *RPA190* SNPs in nine F1 progeny suggested mitotic loss of heterozygosity (conversion) or recombination acting over very short physical genome distances. It is possible that this phenomenon may also have contributed to the skewed segregation of the Mefenoxam insensitivity phenotype in the cross described here, although the SNPs involved do not encode changes in the peptide sequence. Loss of heterozygosity over short physical distances of 700 bp has been observed in *P. sojae* (Chamnanpunt *et al*., 2001; Whisson *et al*., 2004), but the distances observed here within *P. infestans RPA190* ranged from 117 to 378 bp. Interestingly, these loss of heterozygosity events occurred in similar regions of the gene sequence in three of the F1 progeny displaying this phenomenon. Presumably, conversion events could maximize genetic recombination after genetic crossing. The recent sequencing of the *P. capsici* genome has identified loss of heterozygosity (mitotic conversion) across large proportions of that genome, and has been speculated to be a driving force for pathogenic adaptation in that species (Lamour *et al*., 2012a). Our observations of putative conversion events were made in a controlled laboratory cross, and it remains to be shown whether mitotic loss of heterozygosity occurs in field isolates of *P. infestans*.

A definitive test to determine whether a specific allele of a gene leads to a phenotype is to transfer that allele by genetic transformation and observe any change in phenotype. In this study, sensitive and insensitive allele variants of *RPA190* were transformed into sensitive *P. infestans* isolate Ca65. Only transformants that expressed the insensitive allele of *RPA190* grew in the presence of Mefenoxam. All lines expressing introduced alleles of *RPA190* were slow growing, although some other nonexpressing lines also exhibited slow growth. The reason for the slow growth of these transformed lines was not investigated further here. As only a modest number of transgenic lines were produced that expressed either introduced allele of *RPA190*, an alternative interpretation of our results may be that the increased RPA190 levels sequestered all of the available Mefenoxam, allowing endogenous RNApolI activity and hyphal growth. However, this would still indicate that RPA190, and hence RNApolI, is a target for Mefenoxam.

Phytophthora infestans has a technically challenging and flexible genetic system, with genetic analyses complicated by loss of heterozygosity events (this study), and ploidy variations and chromosomal rearrangements (van der Lee *et al*., 2004). The use of crosses to determine the inheritance of Metalaxyl/Mefenoxam insensitivity and to identify linked genetic markers has proven to be difficult in previous studies (Fabritius *et al*., 1997; Judelson and Roberts, 1999; Knapova *et al*., 2002). Multiple genome sequences for *P. infestans* have now been generated (Cooke *et al*., 2012; Haas *et al*., 2009; Martin *et al*., 2013; Yoshida *et al*., 2013), allowing genome-wide identification of SNPs. A strategy to exploit this knowledge, such as genome-wide association mapping (GWAS) (Han and Huang, 2013; Korte and Farlow, 2013), may be a useful adjunct or alternative to inheritance studies to facilitate the identification of other loci contributing to Mefenoxam insensitivity. To avoid any bias in such experiments, it will be essential to consider the population structure of *P. infestans*, as oversampling of clonal lineages may lead to overestimation of SNPs associated with Mefenoxam insensitivity.

The practical application of determining the protein that conditions insensitivity to a control chemical is that DNA sequence-based assays for the rapid determination of insensitivity in field isolates may be developed. This has been carried out for the chemical Mandipropamid to demonstrate that isolates of the downy mildew *Plasmopara viticola* possess the same genetic basis of insensitivity as *P. infestans* (Blum *et al*., 2010b). However, SNPs at multiple sites may also lead to insensitivity, and possibly different levels, such as for the *CYP51* gene in fungi which determines insensitivity to triazole fungicides (Cools *et al*., 2011; Fraaije *et al*., 2007). For Mefenoxam insensitivity in *P. infestans*, sequence-based assessments may need to determine the entire gene sequences for field isolates, or multiplex assays for several specific SNPs. To gain a deeper understanding of sequence diversity among the RNApolI subunits, and hence their potential for contributing to Mefenoxam insensitivity, additional sequencing of the RNApolI subunit genes from a broader range of *P. infestans* genotypes will be essential.

Although durable host plant resistance to oomycete pathogens is a high priority of many crop research programmes, there are oomycete pathogens for which host crop plant resistance has not been identified, is poorly defined or incomplete (Guest, 2007; Lamour *et al*., 2012b; Mahomed and van den Berg, 2011). In these instances, the application of control chemicals will continue to provide security for crop production. The determination of pathogen genes that condition insensitivity to control chemicals offers the possibility to accurately monitor pathogen population changes and respond with changes to disease control strategies. Here, we have identified sequence variation in RPA190 as a component of Mefenoxam insensitivity. This chemical continues to provide control of many oomycete diseases, despite the occurrence of insensitive pathogen genotypes. Our findings may contribute to the sustained use of this control chemical in future.

EXPERIMENTAL PROCEDURES

Phytophthora infestans isolates

Phytophthora infestans isolates (Table 2) are held in the culture collection at the James Hutton Institute, Dundee, UK. T30-4 originated from an F1 population from a sexual cross between isolates 88133 and 80029 (van der Lee *et al*., 1997), and is the reference isolate used for genome

sequencing (Haas *et al*., 2009). Isolates were maintained on rye agar (Caten and Jinks, 1968) amended with pimaricin (10 μg/mL; Sigma, Gillingham, UK) and rifampicin (10 μg/mL; Sigma) at 20 °C in darkness. Transgenic lines of *P. infestans* isolate Ca65 were maintained as above, but agar medium was supplemented with geneticin (G418; 10 μg/mL; Sigma).

Phytophthora infestans cross, oospore germination and determination of F1 progeny

A cross between T30-4 and 88133 was established, and oospores were isolated and germinated as described in Blum *et al*. (2010a). Oospores germinating on water agar were transferred to rye agar (no antibiotics, no Mefenoxam) to develop into colonies. Individual cultures were then tested for the inheritance of AFLP markers from each parental isolate to confirm that these were F1 and not clonal parental type cultures.

AFLP analysis was performed essentially as described by Vos *et al*. (1995), but with minor modifications. DNA (250 ng) was digested with *Eco*RI/*Mse*I, and pre-amplified with nonselective primers. Pre-amplified DNA was diluted 10-fold in water, before amplification with selective primers. After selective PCR with E + 2 and M + 2 primers, samples were not dried prior to the addition of formamide dye [98% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), xylene cyanol, bromophenol blue]. The primer combinations tested were E + AC/M + CT, E + AC/M + GG, E + AA/M + CT and E + AA/M + GG. These primer combinations yielded a total of 31 AFLP markers, with 16 originating from T30-4 and 15 originating from 88133.

Phytophthora infestans sensitivity to Mefenoxam

Preparations of Mefenoxam were supplied by Syngenta Ltd, Cambridge, UK. Sensitivity to Mefenoxam was assessed by growth inhibition of the pathogen on Mefenoxam-amended rye agar plates. Growth of wild-type and transgenic *P. infestans* was tested by measurement of radial hyphal growth on rye agar containing 0, 5 and 100 μg/mL Mefenoxam, as described in Lee *et al*. (1999), except that measurements were taken at 10 days post-inoculation (dpi). Additional Mefenoxam concentrations of 10, 25 and 50 μg/mL were also tested for transgenic lines.

Bioinformatics

Sequences of the RNApolI subunits were recovered by querying the *P. infestans* genome database for RNA polymerase-associated genes (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans). *RPABC10α* and *RPABC10β* were identified by searching the *P. infestans* genome database using TBLASTN (default search parameters) with the *S. cerevisiae* protein orthologues (Kuhn *et al*., 2007) as query sequences. A similar search strategy was used for RPA14 and RPA34.5, but did not return any similar sequences using default search parameters, and so relaxed parameters of $E = 1$ and no filtering were used. Similar searches of other oomycete genome sequences in the National Center for Biotechnology Information (NCBI) GenBank for RPA14 and RPA34.5 were also carried out using the same search parameters. Domains in RPA190 were identified using the *P. infestans* RPA190 sequence to query the Pfam database (version 27.0; http://pfam.sanger.ac.uk/) employing default gathering threshold settings.

Sequencing of RNApolI subunits from *P. infestans*

RNApolI subunits were amplified by PCR from genomic DNA of *P. infestans* using the primers listed in Table S6 (see Supporting Information). The three near-identical paralogues of *RPAC40* were differentially amplified using primer combinations specific to sequences flanking the open reading frames of each paralogue (Table S6). Each 50-μL PCR contained the following components: 0.5 U Phusion Hot Start DNA polymerase (New England Biolabs, Hitchin, UK), 10 μL of 5 × reaction buffer (New England Biolabs), 200 μM deoxynucleotide triphosphates (Promega, Southampton, UK), 600 nM forward and reverse primers, and 30 ng genomic DNA. Thermocycling conditions used an initial melt of 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 3 min (for *RPA190*). The extension time varied, depending on the length of the amplicon, and based on the polymerization of two kilobases of DNA per minute for Phusion polymerase. A final extension step of 72 °C for 10 min was included. PCR products were purified (Minelute kit; QIAGEN, Manchester, UK) prior to sequencing.

DNA sequences of *RPA190* and other RNApolI subunits were determined on a 48 capillary ABI3730 sequencer (Applied Biosystems, Life Technologies, Paisley, UK). Sequences were edited and aligned using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Heterozygous SNPs (oomycetes are at least diploid) identified in the sequence traces were annotated using standard IUPAC nucleotide ambiguity codes.

Transformation of *P. infestans*

The 5.4-kb *RPA190* gene (PITG_03855; GenBank EEY66296), encoding the large subunit of RNApolI, was amplified from genomic DNA of *P. infestans* isolates 80029 (insensitive allele) and 88133 (sensitive allele) using primers POLIClaF (incorporating a *Cla*I site) and POLNotRR (incorporating a *Not*I site) (Table S6). PCR conditions were as described previously. PCR products were purified (Qiagen Minelute kit), digested with *Cla*I and *Not*I restriction endonucleases (Promega), and purified again by Minelute. PCR products were directionally ligated, using T4 DNA ligase (Promega), into the *Cla*I and *Not*I sites of vector pTor (GenBank EU257520) for constitutive expression from the *Bremia lactucae Ham34* promoter. Insert orientation and integrity were confirmed by DNA sequencing.

Transformations of *P. infestans* were carried out using a modified polyethylene glycol–$CaCl_2$–Lipofectin protocol (http://oomyceteworld.net/; Judelson *et al*., 1991). Modifications to this protocol were as described in Grouffaud *et al*. (2008).

RNA isolation and cDNA synthesis

Wild-type isolate Ca65 and transgenic lines were grown in pea broth for 7 days, and mycelium was harvested and frozen at −70 °C until required for RNA isolation. Total RNA for RT-PCR analysis was extracted from frozen mycelium samples using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Prior to cDNA synthesis, all RNA samples were DNase treated using a Turbo DNA-free kit (Ambion, Life Technologies). The yield and integrity of RNA were assessed using a NanoDrop ND-1000 Micro Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. First-strand cDNA for RT-PCR was synthesized from 1 μg of total RNA by

oligo-dT priming using the Superscript II cDNA Synthesis Kit (Invitrogen, Life Technologies), following the manufacturer's protocol, but with the first-strand cDNA synthesis time extended to 2 h.

RT-PCR assays

The *P. infestans RPA190* transgenic copy was specifically amplified using a forward primer within the gene sequence (near the 3' end of the gene) and a reverse primer located within the 3' untranslated region (UTR) pTor vector sequence and preceding the predicted polyadenylation signal (Table S6). Previously designed primers were used to amplify *PiActA* transcripts (actin A; AAA33749; endogenous control) from oligo-dT-primed cDNA. Each 20-μL RT-PCR contained 0.5 U Taq DNA polymerase (Promega), 2 μL of 10 × reaction buffer, 1.5 mM $MgCl_2$, 160 μM deoxynucleotide triphosphates (Promega), 300 nM of each primer and 10 ng of cDNA. Thermocycling conditions were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. A final extension step of 72 °C for 10 min was included. RT-PCR of the full 5.4-kb length of the *RPA190* transgene was also carried out using the POLIClaF forward and pTor UTR reverse primers (Table S6) and Phusion polymerase. The reaction conditions were as described previously.

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SUPPORTING INFORMATION

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

Fig. S1 Growth of *Phytophthora infestans* wild-type Ca65 and lines transformed with constructs containing alleles of *RPA190* on rye agar containing no antibiotics or Mefenoxam.

Table S1 Single nucleotide polymorphisms (SNPs) found in *Phytophthora infestans* RNA polymerase I (RNApolI) subunits.

Table S2 All single nucleotide polymorphisms (SNPs) found in *RPA190* from 15 *Phytophthora infestans* isolates. Heterozygous SNPs are shown as standard nucleotide ambiguity code.

Table S3 Segregation of single nucleotide polymorphisms (SNPs) from *RPA190*, *RPA135* and *RPAC40* in parental isolates and 24 F1 progeny of *Phytophthora infestans* from the T30-4 × 88133 cross.

Table S4 Loss of heterozygosity events in four F1 progeny from the *Phytophthora infestans* T30-4 × 88133 cross.

Table S5 Single nucleotide polymorphisms (SNPs) identified in *RPA190* from 15 *Phytophthora infestans* isolates. SNPs highlighted in pink are found only in insensitive isolates.

Table S6 Oligonucleotides used for amplification and sequencing of RNA polymerase I subunits from *Phytophthora infestans*.

