Mandipropamid targets the cellulose synthase-like PiCesA3 to inhibit cell wall biosynthesis in the oomycete plant pathogen, *Phytophthora infestans*

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SUMMARY

Oomycete plant pathogens cause a wide variety of economically and environmentally important plant diseases. Mandipropamid (MPD) is a carboxylic acid amide (CAA) effective against downy mildews, such as *Plasmopara viticola* on grapes and potato late blight caused by *Phytophthora infestans*. Historically, the identification of the mode of action of oomycete-specific control agents has been problematic. Here, we describe how a combination of biochemical and genetic techniques has been utilized to identify the molecular target of MPD in *P. infestans*. *Phytophthora infestans* germinating cysts treated with MPD produced swelling symptoms typical of cell wall synthesis inhibitors, and these effects were reversible after washing with H₂O. Uptake studies with ¹⁴C-labelled MPD showed that this oomycete control agent acts on the cell wall and does not enter the cell. Furthermore, ¹⁴C glucose incorporation into cellulose was perturbed in the presence of MPD which, taken together, suggests that the inhibition of cellulose synthesis is the primary effect of MPD. Laboratory mutants, insensitive to MPD, were raised by ethyl methane sulphonate (EMS) mutagenesis, and gene sequence analysis of cellulose synthase genes in these mutants revealed two point mutations in the *PiCesA3* gene, known to be involved in cellulose synthesis. Both mutations in the *PiCesA3* gene result in a change to the same amino acid (glycine-1105) in the protein. The transformation and expression of a mutated *PiCesA3* allele was carried out in a sensitive wild-type isolate to demonstrate that the mutations in *PiCesA3* were responsible for the MPD insensitivity phenotype.

INTRODUCTION

Large-scale agriculture involving monocultures of crop plants requires that plant diseases caused by fungi, bacteria, oomycetes and nematodes be controlled by plant disease resistance genes or the application of agrochemicals. In many production systems, resistance genes are overcome by divergent pathogen strains, signifying that chemical application is a necessary component of crop production. Among disease-causing agents, phytopathogenic oomycetes encompass some of the most economically significant pathogens of agricultural crops, such as soybean, vines, peppers and potato. The majority of phytopathogenic oomycetes are obligate parasites and cause great damage and even total crop loss as a result of rapid destruction of the host plant.

Potato (*Solanum tuberosum*) is one of the five most important food crops, producing more starch per hectare than any other crop. Global potato production increased from about 260 million tons in 1991 to some 320 million tons in 2007. The estimates for actual losses in potatoes caused by pathogens worldwide total 14% and, without crop protection, almost 75% of attainable potato production would be lost to pests (Oerke, 2006). Furthermore, the estimated cost, caused by losses and control measures, amounts to over US\$5 billion per year (Duncan, 1999). The phytopathogen primarily responsible for these losses is the oomycete *Phytophthora infestans* (Mont.) de Bary. This pathogen produces a variety of specialized structures prior to host plant penetration, such as heterokont zoospores that are able to swim towards host plant cues and walled cysts that germinate on contact with the host and form appressoria that penetrate the host tissue (Walker and van West, 2007). Zoospores do not have cell walls, but cell wall biosynthesis is a prerequisite for the successful infection of plants. In *P. infestans*, this process takes place during encystment of the zoospores. Further extension and

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thickening of the cell wall occurs during germination of the cyst and subsequent production of the appressorium (Grenville-Briggs *et al.*, 2008). Like all members of the oomycetes, the cell wall of *P. infestans* consists mainly of (1→3)- β -D-glucans, (1→6)- β -D-glucans and cellulose (Bartnicki-Garcia, 1968). After host penetration by appressoria, the pathogen grows inside the host tissue as intercellular hyphae that elaborate intracellular biotrophic haustoria when contact is made with host cells (Avrova *et al.*, 2008). Haustoria and intercellular hyphae secrete effector proteins that act on host cells (Whisson *et al.*, 2007), ultimately leading to the visible symptoms of late blight, a disease lesion with a necrotic and sporulating centre, and a biotrophic margin in which new cells become infected. In order to avoid the yield losses caused by *P. infestans*, control measures are achieved mainly by chemical products. Several chemical classes of oomycete control agent are available, each with varying degrees of systemicity, specificity, duration of activity and risk of resistance. Older oomycete-active fungicides were typically multi-site in action and included dithiocarbamates (e.g. mancozeb), phthalimides (folpet) and chloronitriles (chlorothalonil). More recently, single-site, oomycete-active compounds have been developed with higher potency and a very specific spectrum of activity. The carboxylic acid amide (CAA) group of compounds are one class of single-site molecule displaying effective control of oomycetes and, in particular, *Phytophthora* and *Plasmopara* species. Several molecules, for example iprovalicarb (IPRO), benthiavalicarb, dimethomorph (DMM), flumorph, valifenalate and mandipropamid (MPD), are classified as CAA compounds. MPD has been shown to be highly active against foliar oomycete pathogens, for example *P. infestans*, and *Plasmopara viticola*, the causal agent of grape downy mildew (Huggenberger *et al.*, 2005). *In vitro* growth assays with *P. infestans* have indicated that MPD has no effect on zoospore release from sporangia or on zoospore motility (Knauf-Beiter and Hermann, 2005). Cross-resistance was observed between MPD and all other CAAs in *P. viticola* (Gisi *et al.*, 2007), indicating that these molecules share a similar mechanism of inhibitory activity. Despite numerous attempts, the mode of action of CAAs has, until now, remained unknown. Several studies have described or suggested modifications of the cell wall structure (Cohen *et al.*, 1995, Jende *et al.*, 2001, Reuveni, 2003), modifications in phospholipid biosynthesis (Griffiths *et al.*, 2003) or the disruption of F-actin (Sheng Zhu *et al.*, 2007) as a primary mode of action.

In this study, we describe the molecular mechanism by which MPD inhibits oomycete pathogen growth. The very specific injury symptoms after MPD treatment of *P. infestans* cysts and germ-lings have led us to hypothesize that cellulose synthesis is the main target of this compound. ^{14}C labelling studies of MPD and glucose both indicate that MPD does not enter the *P. infestans* cell, but inhibits cellulose synthesis at the cell wall. For the first time, it has been possible to generate homozygous *P. infestans*

mutants with stable insensitivity to an oomycete-active CAA compound by ethyl methane sulphonate (EMS) mutagenesis. Sequence analysis of the genes responsible for cellulose biosynthesis in sensitive wild-type and insensitive mutant strains have confirmed our hypothesis that MPD inhibits cellulose synthesis. MPD insensitivity was confirmed by the expression of a mutant *PiCesA3* allele in an MPD-sensitive *P. infestans* background.

RESULTS

Symptomatology following treatment with MPD

Symptomatology often yields valuable clues with regard to the effect of a compound on a cell. We therefore took advantage of the fact that *P. infestans* can be induced to differentiate through the preinfection stages of the life cycle *in vitro* (Krämer *et al.*, 1997) to observe MPD-induced symptomatology. Zoospores are released in cold, wet conditions and encystment of these zoospores can be induced by mechanical agitation. Cysts can then germinate and produce appressoria after approximately 16 h. We undertook microscopic studies of germinating cysts with or without treatment with MPD. Germinating cysts were obtained from a zoospore suspension that was vortexed to induce encystment. These cysts were then allowed to germinate and form primary germ tubes.

In untreated *P. infestans*, 80% of the cysts germinated and produced primary germ tubes (Fig. 1A). Treatment with 68 nM MPD for 3 h produced shortened germ tubes as a result of arrested growth and dramatic changes to the germ tube tip with obvious swelling (Fig. 1B). These are not appressoria, as the swelling appears much earlier than the 16 h normally required to produce these structures *in vitro* (Grenville-Briggs *et al.*, 2008).

The swelling of the *P. infestans* germ tube tip is similar to that observed in pollen tubes when treated with isoxaben or with microtubule inhibitors, and in plant roots following treatment with CGA 325'615 (Anderhag *et al.*, 2000; Lazzaro *et al.*, 2003; Peng *et al.*, 2001), suggesting that cell wall structure or microtubule organization may be disrupted. Many cell wall biosynthesis inhibitors, such as isoxaben, display weak sorption to their target, and therefore the effect of the compounds can be readily reversed by washing of treated cells (Hofmannova *et al.*, 2008). To investigate whether MPD also showed similar characteristics, we treated germinating cysts with MPD for 1 h, and then washed the cells three times and allowed recovery for 2 h before observation. As can be seen in Fig. 1C, a 1-h treatment with MPD caused growth inhibition and the appearance of swollen structures. However, washing the compound away resulted in normal germ tube growth and normal hyphal tip emergence from the MPD-induced structures. Furthermore, initiation of germ tube growth was observed at random positions on these swollen structures.

Fig. 1 Morphology of *Phytophthora infestans* germinating cysts treated with mandipropamid (MPD). (A) Germinating cysts after 3 h without MPD treatment produce long germ tubes of uniform diameter. (B) Germinating cysts after 3 h in 68 nM MPD produce germ tubes with swollen tips. (C) Germinating cysts treated with 68 nM MPD for 1 h, washed three times with H₂O and allowed to grow for a further 2 h on a glass slide show resumed normal growth from the previously swollen germ tube tips. Scale bars in all images represent 125 μ m.

To determine whether the observed MPD-induced *P. infestans* symptomatology could be produced by microtubule inhibitors, identical experiments were undertaken with the microtubule inhibitor oryzalin. Oryzalin treatment produced growth inhibition in *P. infestans*. The observed half-maximal effective concentration (EC₅₀) for cyst germination inhibition was 11.6 μ M, but germ tube tip swelling was not observed (data not shown). These data suggest that the observed germ tube swelling is not a result of microtubule disruption.

Chlorophenyl-U-¹⁴C-MPD incorporation into *P. infestans*

In order to have an effect on cell wall biosynthesis, a compound does not necessarily have to be taken up into the cell at high levels. To assess compound uptake, chlorophenyl-U-¹⁴C-MPD was used in cell uptake studies. Germinating cysts were treated with 17, 34 and 68 nM chlorophenyl-U-¹⁴C-labelled MPD and, in each case, the total radioactive incorporation was expressed as 100% (Table 1). In all cases, more than 96.6% of the radiolabel was not retained and was removed with the supernatant; a further 2.44%–2.54% was removed with one H₂O wash. The radioactivity incorporated into the cells was calculated following the subtraction of the cell-free blank (the level of radioactivity retained nonspecifically by the experimental apparatus), leaving only 0.43%, 0.36% and 0% of the initial radioactivity incorporated into the cell fraction in the 17, 34 and 68 nM chlorophenyl-U-¹⁴C-labelled MPD treatments, respectively. These extremely low levels of retention of radioactivity at all tested MPD concentrations suggest that MPD is not taken up by the cell or that MPD mediates its effects at low picomolar concentrations. Interestingly, this low level of uptake and the reversibility of the symptoms by H₂O washing are also characteristics described for plant cellulose synthesis inhibitors (Fisher and Cyr, 1998).

D-(U-¹⁴C)-Glucose incorporation into *P. infestans* cellulose

The cell wall of oomycetes predominantly consists of (1 $\rightarrow$ 3)- β -D-glucans, (1 $\rightarrow$ 6)- β -D-glucans and cellulose, which is composed of (1 $\rightarrow$ 4)- β -D-glucans (Bartnicki-Garcia, 1968). As with plants (Brown, 1996), microfibrillar cellulose or crystalline cellulose is the major constituent of oomycete cell walls (Helbert *et al.*,

Table 1 Incorporation of U-¹⁴C-mandipropamid (U-¹⁴C-MPD) into *Phytophthora infestans* (strain 96).

U- ¹⁴ C-MPD concentration (nM)	Germination inhibition (%)	Incorporation of radiolabel (%)*		
		In supernatant	In wash	In cells
17	36	96.6 ± 1.2	2.54 ± 0.11	0.43 ± 0.02
34	84	96.6 ± 0.4	2.65 ± 0.03	0.35 ± 0.006
68	96	96.8 ± 0.3	2.44 ± 0.38	−0.03 ± 0.04

*Data are mean ± SE (*n* = 3) expressed as a percentage of applied radioactivity.

1997). D-(U-¹⁴C)-Glucose incorporation assays are routinely used to measure *de novo* cellulose biosynthesis in plants (Peng *et al.*, 2001). In this study, we define crystalline cellulose as the fraction of the cell wall that is resistant to digestion with acetic–nitric reagent (Updegraff, 1969), a fraction that has been shown to consist almost exclusively of glucan in a β-1,4-linkage (Meinert and Delmer, 1977). In order to determine whether MPD has an effect on cellulose biosynthesis in *P. infestans*, assays for D-(U-¹⁴C)-glucose incorporation into cellulose were undertaken on germinating cysts.

Cysts were allowed to germinate and form primary germ tubes for 2 h prior to the introduction of D-(U-¹⁴C)-glucose with and without MPD, and incubated for 6 h before the germ tube length was assessed and samples were harvested. D-(U-¹⁴C)-glucose incorporation into untreated germ tubes was seen to be linear over the incubation period and correlated well with germ tube elongation (data not shown). In untreated *P. infestans* cells, 21% of the D-(U-¹⁴C)-labelled glucose was taken up and, after 6 h of incubation, 7% of the radiolabel was incorporated into crystalline cellulose. At higher doses of MPD (68 nM), all biological processes, from germ tube length to glucose incorporation, as well as cellulose synthesis, were reduced. However, at lower doses of MPD (17 nM), glucose incorporation was observed at the same level as in untreated cells and germ tube growth was not affected (Fig. 2). However, at this concentration, crystalline cellulose synthesis was inhibited by 50%. This suggests that the reduction in cellulose biosynthesis is a primary effect of MPD treatment, as this occurs prior to any measurable growth defect. We found that the synthesis of crystalline ¹⁴C-cellulose was inhibited by MPD with a half-maximal inhibitory concentration (IC₅₀) of 19.8 ± 5.6 nM. Taken together with the MPD symptomatology, this result provides evidence that the MPD effect on *P. infestans* is mediated by the inhibition of cell wall cellulose biosynthesis or another cell wall biosynthesis-associated process.

Mutagenesis of *P. infestans* T30-4 leading to MPD insensitivity

Treatment of 8 × 10⁷ protoplasts with EMS, followed by selection on 0.35 μM MPD, yielded 18 putative mutants with elevated insensitivity to MPD. Of these, 13 exhibited only modest

Fig. 2 Effect of mandipropamid (MPD) on cellulose synthesis and germ tube elongation. Effect of MPD on the uptake of D-(U-¹⁴C)-glucose into *Phytophthora infestans* germinating cysts (black bars) and incorporation of radiolabel into cellulose (grey bars). Germ tube growth was evaluated in aliquots of each treatment (hatched bars). The percentage uptake represents the percentage of added radioactivity taken up by the cells. The percentage incorporation is the percentage of radioactivity taken up by the culture, which has been incorporated into cellulose. Data are the means ± SE (*n* = 3).

increases in MPD insensitivity, growing poorly on 0.35 or 0.7 μM MPD (10 × inhibitory concentration), and were fully inhibited by 1.75 μM MPD (25 × inhibitory concentration). In contrast, mutants Tmut3 and Tmut7 exhibited growth on MPD-amended rye agar at concentrations of 1.75 μM. As can be seen in Fig. 3, the growth of Tmut7 was retarded by 35 μM (500 × inhibitory concentration), although growth was still recorded at this concentration, and the growth of Tmut3 was not affected by any MPD concentration tested. The mutant Tmut7 grew more slowly than the wild-type on rye agar without MPD, whereas Tmut3 exhibited wild-type growth in the absence of MPD selection. Both mutants produced typical sporangia which, although fewer in number than generated by wild-type T30-4, produced zoospores that encysted, germinated and grew normally, and pathogenicity was not compromised.

The pairing of mutants with wild-type isolates of A2 mating type showed both to be impaired in the ability to form oospores. That is, matings involving mutants and wild-type A2 tester isolate 88133, as well as other wild-type isolates (see Table 2), produced oospores that were typically nonviable, as assessed by tetrazolium bromide staining. Wild-type matings yielded large

Fig. 3 Sensitivity of wild-type (wt) and mutated *Phytophthora infestans* to mandipropamid (MPD). Sensitivity was assessed by growth inhibition on rye agar containing increasing concentrations (μM) of MPD. The minimum inhibitory dose determined for isolate T30-4 was $0.07 \mu\text{M}$. The maximum concentration shown here ($35 \mu\text{M}$) represents $500 \times$ the minimum inhibitory dose. The growth of mutant Tmut3 was unaffected by any concentration tested, whereas mutant Tmut7 exhibited reduced growth at higher MPD doses. Mutant Tmut7 showed slower growth compared with the wild-type on unamended rye agar.

Table 2 *Phytophthora infestans* strains used in this study.

Strain	Description	Origin
96	Wild-type. Isolated in 1974 from potato in Les Barges, Switzerland. A1 mating type	*
T30-4	Wild-type. F ₁ from a sexual cross between isolates 88133 and 80029. A1 mating type	†
88069	Wild-type. Isolated in 1988 from tomato, the Netherlands. A1 mating type	†
88133	Wild-type. Isolated in 1988 from potato, the Netherlands. A2 mating type	†
96.70	Wild-type. Isolated in 2002 from potato in Wales, UK. A2 mating type	†
550	Wild-type. Isolated in 1983 from potato in Mexico. A2 mating type	†
Mutants		
Tmut3	T30-4 mutagenized by EMS	*†
Tmut7	T30-4 mutagenized by EMS	*†
Transformants		
CES3-1	Wild-type 88069. A1 mating type. Transformed with Tmut3 allele	*†
CES3-4	Wild-type 88069. A1 mating type. Transformed with Tmut3 allele	*†

EMS, ethyl methane sulphonate.

**P. infestans* culture collection of Syngenta Crop Protection, Stein, Switzerland.

†*P. infestans* culture collection at Scottish Crop Research Institute.

numbers of viable oospores that had cell walls of even thickness, and cytoplasm that filled the oosphere (Fig. 4A, B). Nonviable oospores from mutant $\times$ 88133 pairings exhibited empty oospheres, irregular and apparently degenerated cell walls or

condensed granular cytoplasm (Fig. 4C–E). No oospores from any pairing involving mutants germinated and no progeny could be produced to verify their parentage. Additional control matings involving MPD-insensitive mutants and wild-type A1 tester isolates T30-4 and 88069 did not produce any oospores, signifying that neither EMS mutagenesis nor growth on MPD medium induced any change in mating type.

Sensitivity of Tmut3 and Tmut7 mutants to other CAAs

Several other CAA compounds have been used to control late blight, such as DMM and IPRO. In order to determine the relative sensitivity of the mutants generated in this work to other CAAs, the germ tube length was assessed and IC_{50} values were determined for MPD, DMM and IPRO in the T30-4 wild-type, Tmut3 and Tmut7 mutants (shown in Table 3). MPD was the most potent compound controlling wild-type *P. infestans* growth, followed by IPRO and DMM. The sensitivity shifts observed for both mutants were of the same order of magnitude; precise IC_{50} values could not be determined accurately for the mutants, as compound solubility limits were reached for MPD and IPRO in the test system. The relative sensitivity shift was the highest for MPD, with a shift of over 1500-fold. Such a large shift in sensitivity is characteristic of a mutation in a compound target site (Sierotzki and Gisi, 2003). Interestingly, IPRO and DMM also showed a sensitivity shift, but to a lesser extent for DMM in

Fig. 4 Structures of oospores originating from sexual crosses between wild-type A2 isolate 88133 and Tmut3. Oospores formed *in situ* in mating cultures of *Phytophthora infestans* wild-type A2 testers with wild-type A1 T30-4 or mandipropamid (MPD)-insensitive mutants. (A, B) Oospores from mating cultures of T30-4 and 88133. Oospores are typically abundant, thick-walled and with cytoplasm filling the oosphere. (C–E) Oospores from mating cultures of Tmut3 and 88133. Oospores are sparsely distributed, irregular (E) or thin-walled (D), and cytoplasm is condensed/granular or absent (C). Scale bars in all images represent 20 μ m.

Table 3 Sensitivity evaluation of wild-type and ethyl methane sulphonate (EMS)-generated mutants to different carboxylic acid amides (CAAs).

	Wild-type T30-4		Mutant Tmut3		Mutant Tmut7	
	Germ tube length* (μ m)	Resistance factor	Germ tube length* (μ m)	Resistance factor	Germ tube length* (μ m)	Resistance factor
MPD	0.08 $\pm$ 0.02	1	>121†	>1512	>121†	>1512
DMM	2.3 $\pm$ 0.2	1	87 $\pm$ 24	38	12.1 $\pm$ 2.5	5.3
IPRO	1.4 $\pm$ 0.3	1	>150†	>107	>150†	>107

DMM, dimethomorph; IPRO, iprovalicarb; MPD, mandipropamid.

*Data are IC₅₀ mean $\pm$ SE (n = 3).

†Compound reached limit of solubility.

these mutants. However, the data clearly show that the mutants generated have reduced sensitivity to all three CAA compounds.

Both Tmut3 and Tmut7 mutant strains carry mutations in *PiCesA3*

Oospores produced when mutants Tmut3 and Tmut7 were mated to wild-type A2 tester isolate 88133 were nonviable, and so we were unable to genetically map the mutant alleles, or subsequently perform positional cloning of the gene(s) conditioning MPD resistance. However, the data obtained from phenotypic analysis and D-(U-¹⁴C)-glucose incorporation suggested that the target of MPD was associated with cellulose biosynthesis or another cell wall biosynthesis-associated process. We therefore adopted a candidate gene strategy and sequenced a range of genes involved in cell wall synthesis or remodelling of the cell wall, including the four *PiCesA* genes, which were assumed to be cellulose synthases in *P. infestans* (Grenville-Briggs *et al.*, 2008), a putative endo-1,3- β -glucanase, a glycolipid anchored surface protein that has been shown to exert glucanosyltransferase activity (Carotti *et al.*, 2004), the secretory protein OPEL that was isolated as a putative polygalacturonase

(Meijer *et al.*, 2006), a cell 5A endo-1,4- β -glucanase, and a β -glucosidase/xylosidase exhibiting glycoside hydrolase activity (Brunner *et al.*, 2002). Except for *PiCesA3*, all sequenced candidate genes from the mutants Tmut3 and Tmut7 were identical at the amino acid level to the wild-type T30-4. For the *PiCesA3* gene, sequencing revealed that the gene sequence of the EMS mutants was identical to the wild-type gene, except for a G to T transversion at nucleotide (3417) in Tmut3 and a G to C transversion at the same nucleotide position in Tmut7, located towards the 3' end of the *PiCesA3* coding sequence (Fig. 5). Analysis of the chromatograms indicated that EMS mutagenesis created homozygous mutations in *PiCesA3* in both mutant strains, even though *P. infestans* is a diploid organism (Fig. S1, see Supporting Information). The point mutation in the Tmut3 mutant leads to replacement of a conserved glycine at position 1105 in the *PiCesA3* protein with a valine residue. The *PiCesA3* point mutation in Tmut7 results in replacement of the same conserved glycine residue at position 1105 with an alanine residue (Fig. 5). Both mutations are located in the last of the nine predicted transmembrane domains (Grenville-Briggs *et al.*, 2008) of the *PiCesA3* protein (Fig. 5). Glycine-1105 seems to be conserved among all oomycete *CesA* proteins identified so far.

Fig. 5 Structure and site of mutations to mandipropamid (MPD) insensitivity in the *PiCesA3* gene. (A) Intron/exon structure of the *PiCesA3* gene. Numbers correspond to the size in base pairs. Point mutations in Tmut3 and Tmut7 and the predicted amino acid exchanges in the mutant gene products are indicated. (B) Alignment of the amino acid sequence of CesA1, CesA2, CesA3 and CesA4 between *Phytophthora infestans*, *P. ramorum* and *P. sojae* at the site of mutation to MPD insensitivity. Gly1105 is conserved in each of the four CesA genes.

Alignment of the amino acid sequence from 12 CesA proteins of three different organisms (*P. infestans*, *P. ramorum* and *P. sojae*) showed the conserved glycine at position 1105 in each case (Fig. 5).

Back-transformation of *PiCesA3*

The allele of *PiCesA3* from Tmut3 was selected for the complementation of wild-type *P. infestans* isolate 88069 to MPD insensitivity as this mutant exhibited the highest level of insensitivity. Isolate 88069 was selected for transformation as it is amenable to stable transformation. Ten stable transformants were obtained with the Tmut3 *PiCesA3* allele, two control transformants containing only the empty vector, as well as 29 transformants containing the wild-type *PiCesA3* allele. Transformants were assessed for growth on rye agar amended with MPD, and two transformants (ces3-1 and ces3-4) reproducibly exhibited reduced levels of sensitivity to MPD (Fig. 6). However, the reduced sensitivity was not as high as that found for the EMS mutants. In these transformants, the wild-type allele is still present and is likely to be producing PiCesA3 protein that is

sensitive to the effects of MPD. This is likely to compromise the level of shift in sensitivity to MPD in these strains, whereas, in the EMS mutants, only mutated PiCesA3 protein will be formed as only the mutated allele is present in these strains (Table 4). Of these, transformants ces3-1 and ces3-4 were selected for further characterization. The wild-type *PiCesA3*-1105G allele was also introduced into wild-type isolate 88069, and 29 transformants were recovered and tested on 0.07 and 0.7 μ M MPD in rye agar. All 29 transformants exhibited growth inhibition at 0.07 μ M MPD, and none of the 29 transformants grew at 0.7 μ M MPD (Fig. 6), further supporting the role of the *PiCesA3*-1105V allele in conditioning MPD insensitivity.

PiCesA3 gene copy number and expression level in *P. infestans* wild-type strains, EMS mutants and transformants

Southern blot analysis was performed using genomic DNA purified from the wild-type strains T30-4 (wild-type strain used for EMS mutagenesis) and 88069 (wild-type strain used for transformation), and from the two MPD-insensitive mutants Tmut3

Fig. 6 Sensitivity of wild-type and transformed *Phytophthora infestans* to mandipropamid (MPD). Sensitivity was assessed by growth inhibition on rye agar containing increasing concentrations of MPD. Growth is expressed as millimetres of radial expansion. The minimum inhibitory dose determined for all isolates was $0.07 \mu\text{M}$. (A) Transformants with the *PiCesA3* Tmut3 mutated allele were assessed for sensitivity to MPD. The maximum concentration at which growth was recorded ($2.5 \mu\text{M}$) represents $35.7 \times$ the minimum inhibitory dose, whereas the maximum MPD concentration tested here ($25 \mu\text{M}$) represents $357 \times$ the minimum inhibitory dose. Transformants CES 3-1 and CES 3-4 showed clear shifts in MPD sensitivity. (B) Transformants with the wild-type *PiCesA3* allele were assessed for sensitivity to MPD. The maximum MPD concentration is $0.7 \mu\text{M}$ and no transformant expressing the wild-type *PiCesA3* allele grew at this concentration. Black bars, MPD-free medium; white bars, $0.07 \mu\text{M}$ MPD; grey bars, $0.175 \mu\text{M}$ MPD; checkered bars, $0.35 \mu\text{M}$ MPD; hatched bars, $0.7 \mu\text{M}$ MPD; crossed bars, $2.5 \mu\text{M}$ MPD.

Table 4 *PiCesA3* gene copy number and expression levels in *Phytophthora infestans* wild-type (wt) strains, mutants and transformants.

Strain	(G)	(T)	(C)	Copy number	Confidence interval	Relative expression	Confidence interval
T30-4*	98.6			2	2–2	1	0.8–1.2
Tmut3*		99.1		2	2–2	1.3	1.1–1.5
Tmut7*			91.9	2	2–2	0.7	0.6–0.7
88069*	98.4			2	2–2	1	0.9–1.2
pTOR-1*	97.7			2	2–2	0.7	0.6–1.0
ces3-1†	40.8	58.7		3	2.77–2.98	3.2	2.7–3.9
ces3-4†	20.1	79.6		8	7.19–8.79	3.7	2.2–6.2

Tmut3 and Tmut7 were derived from T30-4 by ethyl methane sulphonate (EMS) mutagenesis. ces3-1 and ces3-4 are transformants obtained from 88069 as described in Experimental procedures.

*Copy number was based on Southern blot data; percentage of wild-type (G)/mutant (T/C) allele obtained by pyrosequencing.

†Copy number of mutant *PiCesA3* allele was deduced by relative quantification of pyrosequencing peak signals between *PiCesA3* wild-type (G) and *PiCesA3* mutant (T).

and Tmut7. As shown in Supporting Information (Fig. S2), all strains carry two copies of the *PiCesA3* gene, as expected for a diploid organism.

The mutant *PiCesA3* gene copy number in transformants ces3-1 and ces3-4 was assessed by pyrosequencing. Using the percentage of wild-type (G)/mutant (T/C) allele (*PiCesA3*/Tmut3 *PiCesA3* allele) obtained by pyrosequencing, and assuming that two wild-type genocopies of *PiCesA3* are still present in the transformed strains, we predicted the integration of three and eight mutated copies in ces3-1 and ces3-4, respectively (Table 4). In addition to Sanger sequencing, pyrosequencing was used to confirm the homozygosity of mutated *PiCesA3* genes in both Tmut3 and Tmut7 strains (Table 4). The results confirmed the presence of a unique homozygous allele in each of the strains.

The expression levels of the introduced mutant allele, relative to the background expression of the wild-type allele, were also assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Similar to the copy number analysis, the insensitivity of transformants to MPD was associated with elevated levels of expression of the introduced mutant allele of *PiCesA3* (Table 4).

DISCUSSION

We have identified the protein target of the recently developed and commercialized anti-oomycete agrochemical, MPD. We used a combination of phenotypic observations, inhibitors, incorporation assays, mutation of *P. infestans*, candidate gene sequencing and complementation in *P. infestans* transformants to show that MPD targets the cellulose synthase-like *PiCesA3*. A feature distinguishing oomycetes from true fungi is the presence of cellulose and the low percentage of chitin in their cell walls (Bartnicki-Garcia, 1968; Hardham, 2007). This observation has been confirmed by gene silencing and inhibitor studies, demonstrating that cellulose synthesis is required for normal appressorium formation and infection in *P. infestans* (Grenville-Briggs

et al., 2008). Oomycetes also form coenocytic hyphae and are diploid for the major part of their life cycle. These and other differences (Hyde *et al.*, 2008; Whitaker *et al.*, 2009) can partly explain why oomycetes are not controlled by many of the chemicals used in agriculture to prevent yield losses caused by fungal damage to host plants. Following MPD treatment, *P. infestans* cyst germ tubes display a characteristic symptomatology: growth arrest and swelling of the germ tube tip. The symptomatology observed after MPD treatment is reminiscent of the disruption of cellulose biosynthesis by treatment with various chemical inhibitors that result in a rapid loss of anisotropic growth (Desprez *et al.*, 2002; Scheible *et al.*, 2001). Furthermore, similar symptomatology was observed when the simultaneous silencing of all four putative cellulose synthase genes from *P. infestans* was carried out. In that study, germ tube tips became swollen and formed several successive abnormal appressoria-like structures, or were incapable of constraining cellular turgor pressure, leading to cell lysis (Grenville-Briggs *et al.*, 2008). MPD-treated cells also appeared to be incapable of constraining turgor pressure; however, the tips increased in volume rather than undergoing lysis. This suggests that cell wall remodelling factors remain functional following MPD treatment and that membrane integrity is maintained. Hyphal integrity is likely to be maintained by a combination of the cytoskeleton components, such as actin filaments, tubulin microtubules and cell wall polymers. Disruption of any of these components by MPD may potentially lead to hyphal swelling (Lazzaro *et al.*, 2003). Oryzalin, an inhibitor of plant microtubule polymerization, reduced *P. infestans* cyst germination, but displayed different symptoms from MPD, indicating that the cell wall structure impairment produced by MPD is likely to be via a mechanism other than the inhibition of microtubule polymerization.

The growth reversion observed following a H₂O washing step showed that the cells remain viable after a 1-h treatment at high doses of MPD (68 nM). We also observed that MPD does not significantly penetrate into the cell, indicating that its lethal

effects are a result of targeting a molecule at the cell surface. Moreover, such growth reversion and limited cell uptake have also been described in several plant cell types for the inhibitors isoxaben and CGA 325'615 (Fisher and Cyr, 1998; Peng *et al.*, 2001).

In plants, cellulose microfibrils constitute the major load-bearing structures within the cell wall (Carpita and Gibeau, 1993; McCann and Roberts, 1994). Isoxaben is a cellulose biosynthesis inhibitor in plants and the symptomatology produced by this molecule is strongly reminiscent of that produced by MPD; therefore, we investigated whether their modes of action were analogous and whether *de novo* cellulose synthesis of *P. infestans* was impaired by MPD. The synthesis of crystalline ¹⁴C-cellulose was inhibited by MPD with an IC₅₀ of 19.8 ± 5.6 nM (Fig. 2). Interestingly, the potency of MPD is comparable with that of isoxaben which also inhibits cellulose synthesis when applied in low nanomolar concentrations (Heim *et al.*, 1990). Moreover, the inhibition of cellulose synthesis appears to be a primary effect of MPD, as concentrations of MPD that inhibit D-(U-¹⁴C)-glucose incorporation into the cellulose fractions do not inhibit glucose uptake or germ tube growth. Taken together, these observations led us to postulate that MPD interferes with crystalline cellulose synthesis.

In order to further define the molecular target of MPD, we generated mutants in *P. infestans* that were strongly reduced in sensitivity to MPD. Previous attempts to generate such mutants by UV irradiation or EMS mutagenesis of zoospores have generated mutants marginally insensitive or with fitness penalties (Rubin *et al.*, 2008; Stein and Kirk, 2004). Similar mutation strategies have also failed to generate mutants to DMM (Young *et al.*, 2001; Zhu *et al.*, 2008). As an alternative, we performed EMS mutagenesis on protoplasts of *P. infestans*, followed by cell wall regeneration and selection of mutants on MPD-amended medium.

The recovered mutants Tmut3 and Tmut7 were able to grow on medium containing up to 500-fold lethal doses of MPD. Measurement of germ tube elongation identified loss of sensitivity factors of more than 2000-fold. Such large sensitivity shifts are typically found when target mutations have occurred (Sierotzki and Gisi, 2003). Reduced sensitivity was also observed towards IPRO and DMM; therefore, all three molecules may share a similar target site. The smaller sensitivity shift observed for DMM indicates a slightly different binding mode for DMM when compared with MPD or IPRO.

Although recent advances have been made in the generation and detection of mutant alleles in oomycetes (Lamour *et al.*, 2007), mutagenesis studies have not been widely used as a result of a combination of their diploid nature and frequent difficulties in obtaining homozygous lines for genetically recessive mutant alleles. The sexually derived spores of oomycetes are frequently recalcitrant to germination and, for outcrossing

species such as *P. infestans*, conversion to homozygosity requires the identification of rare lines derived from the self-fertilization of heterozygous mutants. The mutagenesis of oomycetes to drug insensitivity has frequently led to a loss or disruption of one or more life cycle stages, pathogenicity or nutritional impairment (Bruin and Edgington, 1982; Castro *et al.*, 1970; Long and Keen, 1977). Here, mutants to MPD were able to form all asexual stages, and were pathogenic on potato. The mutant Tmut7 exhibited a slight growth penalty, but Tmut3 was indistinguishable from the wild-type T30-4 isolate *in vitro*. The only life cycle stage to show impairment in both mutants was the formation of oospores, negating any attempts at determining the genetic status (recessive/dominant) of the mutation. The oospore defect phenotypes for both mutants may be a genuine effect of the mutation to MPD insensitivity, although this must be reconciled against the low percentage of cellulose present in oospore walls (Lippman *et al.*, 1974). It has been shown that exposure to the anti-oomycete phenylamide compound mefenoxam or benomyl fungicide can lead to changes in mating type in *P. infestans* (Groves and Ristaino, 2000). Here, we found no evidence that either EMS mutagenesis or the growth of mutants on MPD-amended medium resulted in a change in mating type.

Cell walls are highly dynamic structures. Cell shape is strongly influenced by the organization and plastic extensibility of the cell wall. On deposition, cell wall polymers are integrated into existing structures and undergo extensive remodelling during cell expansion (Gonneau *et al.*, 2007). These modifications involve a wide array of cell wall-modifying enzymes, such as expansins, endo-glucanases, polygalacturonases, peroxidases and various glycosidases, which are localized in the cell wall or anchored in the plasma membrane of the cells. Several of these proteins have been identified in the cell walls of oomycetes (Bouzenzana *et al.*, 2006; Meijer *et al.*, 2006). In taking a candidate gene strategy to identify the protein target for MPD, we sequenced the genes encoding a range of such enzymes in wild-type and MPD-insensitive mutants. No mutations were found in these genes.

Cellulose microfibrils are synthesized at the plasma membrane from hexameric complexes that contain cellulose synthase catalytic units (CESA) (Doblin *et al.*, 2002). These complexes are composed of three non-redundant Cesa isoforms. In *Arabidopsis thaliana*, the Cesa family comprises 10 genes, and at least 21 genes in rice. In addition to these 'classical' Cesa genes, 29 CSA-like (CSL) genes have been found that are related to Cesa, but the function of which has not been clearly elucidated (Gonneau *et al.*, 2007). However, in *Nicotiana glauca* pollen tubes, a CSL gene is abundantly expressed during pollen tube growth, whereas no Cesa gene expression was detected (Doblin *et al.*, 2001).

In contrast with the complexity of Cesa and CSL genes in plants, *P. infestans* possesses four cellulose synthase genes (Cesa) that have been functionally characterized (Grenville-

Briggs *et al.*, 2008). *PiCesA1*, *PiCesA2* and *PiCesA4* form a distinct phylogenetic group of genes that are closely related to cellulose synthases from cyanobacteria. *PiCesA3*, although containing the signature (QXXRW) of a glycosyl transferase, is more similar to CSL genes than to true CesA genes. In the sequencing of candidate genes conditioning MPD insensitivity, the *PiCesA3* genes from the mutant strains were found to have single nucleotide polymorphisms (SNPs) encoding a G1105V substitution in Tmut3 and a G1105A substitution in Tmut7. This glycine is a highly conserved amino acid within the final predicted transmembrane domain across a range of oomycetes (*P. ramorum*, *P. sojae* and *P. infestans*). Together with the biochemical and symptomatology data, this strongly suggests that the *P. infestans* *PiCesA3* protein is the target of MPD. For definitive confirmation, we undertook a reverse genetic approach, transforming a mutant *PiCes3A* allele into a wild-type *P. infestans* strain. The expression of the mutant *PiCes3A* allele resulted in a loss of sensitivity to MPD. Moreover, this reduced sensitivity was not observed when additional copies of the wild-type allele were introduced. Taken together, these observations lead us to conclude that the *PiCesA3* protein is the target of MPD.

The data presented here also suggest that the mutations in the *PiCesA3* gene which confer reduced sensitivity to MPD are not dominant in *P. infestans*. Two observations support this supposition. Firstly, in EMS mutagenesis experiments, despite repeated screening of very large numbers of mutagenized cells, only homozygous mutants have been found to display reduced sensitivity to MPD. Furthermore, it is notable that, in *A. thaliana*, EMS mutagenesis produced a significant percentage of homozygous mutants, whereas UV mutagenesis did not (Greene *et al.*, 2003), which may explain why UV mutagenesis of *P. infestans* failed to generate mutants with significantly reduced MPD sensitivity. Secondly, further supporting the genetic nondominance of MPD insensitivity, back-transformation experiments yielded a shift in sensitivity that was not as high as that seen in EMS mutagenesis strains, despite a higher copy number and overexpression of the mutant *PiCesA3*1105V allele. In these transformants, we cannot exclude the possibility that sensitive protein is still generated, allowing the MPD effect to be displayed. One hypothesis that would explain the recessive character of the *PiCesA3* mutant gene has been suggested by protein modelling, which predicts that the *PiCesA3* proteins dimerize to form a functional rosette seen in other cellulose synthase enzymes. The dimerization forms a pore through the plasma membrane required for the extrusion of the cellulose polymers. The amino acid change encoded by the mutation could alter the accessibility of MPD to the catalytically active loop exposed to the cytoplasm. The steric changes promoted by this amino acid modification would block the penetration of MPD through the pore of the complex to its binding site, and this model would be consistent with the necessity of having a homozygous mutation.

Such a model is also consistent with the observation that only overexpressing back transformants exhibit any insensitivity to MPD. Mutations in the C-terminal transmembrane region of *A. thaliana* have been shown to affect the interaction and formation of the functional cellulose synthase rosette/complex (Daras *et al.*, 2009). We therefore cannot formally rule out the possibility that the loss of sensitivity to MPD could also be explained by a similar mechanism.

The application of agrochemicals to control disease caused by fungi and oomycetes is presently a major component of plant disease control strategies. Although disease-resistant crop cultivars are being developed, some utilizing genetic modification, there will remain a core role for the chemical control of plant disease, as it is unlikely that plant resistance will control disease caused by all fungi and oomycetes. Although many of the compounds presently available exhibit acceptable environmental and organism toxicity profiles, coupled with high specificity, their mode of action remains to be determined. Understanding the mode of action for a control chemical can assist the rapid screening of further oomycete-active compounds to determine novel modes of action. This report represents the first time that a combination of forward and reverse genetics has been used in oomycetes to identify the target of a control chemical. The application of the strategies used here will aid in the development of new compounds to combat the enormous impact of oomycete-incited plant disease on global food security.

EXPERIMENTAL PROCEDURES

Phytophthora infestans strains and cultures

Cultures of *P. infestans* isolates are held in the culture collection at the Scottish Crop Research Institute, and have been described previously (Armstrong *et al.*, 2005), or are held in the Syngenta culture collection (Syngenta, Stein, Switzerland) as described previously (Dahmen *et al.*, 1983). All strains used for this study are listed in Table 2.

T30-4 originated from an F₁ population from a sexual cross between isolates 88133 and 80029, and is the reference isolate used for genome sequencing (Haas *et al.*, 2009; http://www.broad.mit.edu/annotation/genome/phytophthora_infestans). Isolates were maintained on rye agar (Caten and Jinks, 1968) amended with pimarin (10 µg/mL; Sigma-Aldrich, Buchs, Switzerland) and rifampicin (30 µg/mL; Sigma-Aldrich, Buchs, Switzerland) at 20 °C in the dark.

Active ingredients and derivatives

MPD, IPRO (an amino acid amide) and DMM (a cinnamic acid amide) were supplied and dissolved in dimethyl sulphoxide (DMSO) by Syngenta Crop Protection, Stein, Switzerland.

Chlorophenyl-U-¹⁴C-labelled MPD was dissolved in acetonitrile at a concentration of 2.2 GBq/mmol and was supplied by Syngenta Crop Protection, Basle, Switzerland.

Symptoms on germinating cysts after MPD treatment

Germinating cysts for growth tests were obtained from *in vitro* sporangia formed on 12-day-old agar cultures. These were flooded with H₂O at 4 °C and scraped from the surface of the plates immediately. The suspension was filtered through 50-µm Nylon mesh to remove mycelial fragments and incubated in Petri dishes for 45 min at 4 °C in the dark. For zoospore release, the suspension was incubated at 19 °C for a further 20 min. Zoospores were decanted and filtered through a 20 µm paper filter (Whatman International Ltd, Maidstone, Kent, UK). To induce cyst formation, the zoospore suspension was vortexed for 30 s (Vortex Genie2, Scientific Industries, Bohemia, NY, USA) and incubated for 15 min at 19 °C. Newly formed cysts germinated at 19 °C after incubation for 1–2 h. For the assessment of symptoms induced by MPD, a 500 µL suspension containing 1-h-old germinating cysts (200 000/mL) was transferred to a microcentrifuge tube (2 mL) and mixed with MPD to give the required final concentration of compound. After incubation for 3 h at 19 °C on a shaker (140 rpm), triplicate samples (20 µL) were transferred onto microscopic slides and visualized using a Zeiss (Feldbach, Switzerland) Axiolab light microscope.

For MPD washing experiments, germlings were treated as above with MPD. Incubation was followed by centrifugation for 3 min at 2250 *g* (Eppendorf), the supernatant was decanted, the remaining cells were washed with 1 mL of H₂O, and briefly vortexed. Germinating cysts were centrifuged again and mixed with 300 µL of H₂O. Microscopic analysis was undertaken in triplicate.

Phytophthora infestans sensitivity to MPD

Sensitivity to MPD was assessed by growth inhibition of the pathogen on MPD-treated agar plates, as well as by *in vitro* assays based on measurements of germ tube length elongation in the presence of MPD. To identify the inhibitory dosage for agar cultures, the growth of wild-type T30-4 was tested by measurement of radial hyphal growth at 7 days post-inoculation (dpi) on rye agar containing increasing concentrations of MPD: 0.01–0.1 µM. The growth of cultures insensitive to MPD after EMS mutagenesis was tested by the measurement of radial hyphal growth at 7 dpi on agar containing increasing concentrations of MPD: 0.07–35 µM. The growth of transgenic cultures expressing a mutant allele of the *PiCesA3* gene was measured at 7 dpi as above on increasing concentrations of MPD: 0.07–25 µM.

For *in vitro* cyst germination assays, concentrations from 0.8 to 199 nM were used for the sensitive wild-type T30-4. For

insensitive mutants, MPD concentrations between 124 nM and 121 µM were used. Cysts were prepared as described previously. Cysts were treated with MPD immediately after vortexing, transferred onto microscope slides and incubated for 3 h at 19 °C in the dark. Germ tube length analysis was performed as described previously.

U-¹⁴C-MPD incorporation into *P. infestans* cells

MPD uptake was measured by determining the amount of chlorophenyl-U-¹⁴C-labelled MPD incorporated into *P. infestans* wild-type cysts (T30-4). Three different final concentrations of MPD (17, 34, 68 nM) were used in triplicate. A blank evaluation without cells was undertaken to assess nonspecific binding and was run in duplicate. The cyst suspension (10 mL; 200 000 cysts/mL) was mixed with 50, 100 and 200 µL U-¹⁴C-labelled MPD (3.4 µM), and incubated for 4 h at 20 °C, with slow agitation (120 rpm). Following centrifugation for 6 min at 2500 *g* on a Rotana/RP centrifuge (Hettich, Tuttlingen, Germany), the supernatant (10 mL) was mixed with 10 mL of Intra-Gel Plus universal liquid scintillation cocktail (Packard Bioscience BV, Groenningen, the Netherlands) and analysed using a 2500TR liquid scintillation analyser (Packard Bioscience BV). The remaining cells were washed with 10 mL of distilled H₂O for 10 min, briefly vortexed and centrifuged for 6 min at 2500 *g*. The supernatant was analysed as described before. Washed cells were dissolved in 2 mL of 100% methanol–H₂O (4 : 1) and sonicated for 5 min at 50 °C. The supernatant was mixed with an equal volume of scintillation solution and the radioactivity was measured as above.

D-(U-¹⁴C)-Glucose incorporation into germinating cysts and cellulose

Phytophthora infestans germinating cysts for D-(U-¹⁴C)-glucose incorporation assays were obtained as follows. D-(U-¹⁴C)-glucose (1.85 GBq/mmol) was supplied by GE Healthcare (Basel, Switzerland). D-(U-¹⁴C)-Glucose incorporation was investigated in the presence of different MPD concentrations (17, 34 and 68 nM). For each concentration, triplicates were carried out and the results were quantified in relation to the untreated control. Cyst suspension (0.5 mL; 200 000/mL) was transferred into Corex tubes (15 mL) and incubated for 2 h with slow shaking (120 rpm). After incubation, 5 µL of D-(U-¹⁴C)-glucose (18.5 MBq/mL) and 5 µL MPD (to give the required final concentration) were added. Following incubation for 6 h on a shaker (120 rpm) at 19 °C, the tubes were centrifuged for 2 min in a Rotana/RP centrifuge (Hettich) at 2500 *g*; 250 µL of the supernatant was counted for radioactivity using Insta-Gel Plus (Packard Bioscience BV) in a Packard 2500TR liquid scintillation counter, and this corresponded to the uptake of radiolabel. Quench correction was made automatically by the external stan-

dard channel ratio method. To the remaining fraction (250 µL), including germlings, 50 µL of 10 : 1 acetic–nitric reagent solution (CH₃COOH 80%/HNO₃ 65%; Updegraff, 1969) was added to stop the incorporation process. The suspension was vortexed and incubated overnight. After centrifugation for 7 min at 2500 *g*, the supernatant was decanted and the pellets were dissolved in 5 mL of acetic–nitric solution. The mixture was heated for 1 h at 90 °C, cooled and filtered through Durapore membrane filters (0.45 mm HV, Millipore, Watford, UK). Crystalline cellulose representing the remaining cell wall was washed again with 2 mL of acetic–nitric solution and rinsed three times with 5 mL of H₂O. To determine the radioactivity in the cellulose fraction, dry filters with the remaining crystalline cellulose cell walls were transferred into 15-mL vials, 5 mL of scintillation solution was added and the radioactivity was counted as above.

Mutagenesis of *P. infestans* T30-4 to MPD insensitivity

Protoplasts generated from axenically cultured mycelium, as described by Whisson *et al.* (2005), were used to determine the toxicity of EMS (Sigma-Aldrich, Buchs, Switzerland) to *P. infestans*. Protoplasts (5 mL) were mixed with increasing concentrations of EMS (0.05–0.4 M) in osmoticum (1 M mannitol, 7 mM MgSO₄) for 3 min, and then diluted with osmoticum to 50 mL and collected by centrifugation at 700 *g*, and the protoplasts were allowed to regenerate in 20 mL of pea broth (Whisson *et al.*, 2005) containing 1 M mannitol for 24 h at 20 °C. Regenerated protoplasts were spread evenly onto rye agar plates, incubated at 20 °C for 5 days, and the colonies were counted. Colonies were reliably recovered only from treatments with 0.05 M EMS. This concentration corresponded to approximately 80% lethality and was used in scaled up experiments. Regenerated protoplasts were selected on rye agar amended with 0.35 µM MPD. Colonies insensitive to this concentration appeared from 10 to 14 days after application of selection; these colonies were maintained on medium containing 0.35 µM MPD. Genetically pure cultures of putative mutants were obtained by the generation of cultures originating from single uninucleate zoospores of mutants.

Phenotypic characterization of mutants

Isolates of opposing A1 and A2 mating types were paired on rye agar as strips of agar culture placed approximately 10 mm apart. Oospore formation was observed *in situ* from 7 dpi using an inverted microscope. Oospores were also purified from the mating cultures by excision of the interaction zone from the agar, maceration with sterile distilled H₂O at full speed in a domestic blender, sieving through 50-µm Nylon mesh (Cell Micro Sieves; BioDesign Inc., Carmel, NY, USA), and centrifugation at 700 *g* for

5 min. Isolated oospores were examined directly by light microscopy as above, or stained using tetrazolium bromide staining (Sutherland and Cohen, 1983).

Bioinformatics

Sequences of cellulose synthases were taken from the GENBANK/EMBL/uniprot data libraries and can be found under the following accession numbers: ABP96902 (*PicesA1*), ABP96903 (*PicesA2*), ABP96904 (*PicesA3*), ABP96905 (*PicesA4*), ABP96906 (*PscsA1*), ABP96907 (*PscsA2*), ABP96908 (*PscsA3*), ABP96909 (*PscsA4*), ABP96910 (*PrcesA1*), ABP96911 (*PrcesA2*), ABP96912 (*PrcesA3*), ABP96913 (*PrcesA4*), L38609 (*Agrobacterium tumefaciens celA*) and B0J0N9 (*Rhizobium leguminosarum celA*). Other candidate gene sequences were found at the Broad Institute URL for the *P. infestans* genome sequence under accession numbers PITG_00219 (putative endo-1,3-β-glucanase), PITG_14141 (glycolipid anchored surface protein GAS1), PITG_05156 (secretory protein OPEL), PITG_08611 (cell 5A endo-1,4-β-glucanase) and AF352032 (β-glucosidase/xylosidase).

Nucleic acid manipulations

Total DNA of *P. infestans* was isolated from mycelia following the method of Judelson (1996). PCR was performed as described by Saiki *et al.* (1988) with a T-Gradient Thermocycler (Biometra, Goettingen, Germany) and synthetic oligonucleotide primers (Microsynth, Balgach, Switzerland) (Table S1, see Supporting Information). PCR fragments to be cloned into plasmids were amplified with Platinum Taq Polymerase High Fidelity (Invitrogen, Basel, Switzerland), purified with Sephadryl S-300 (GE Healthcare) and cloned into the vector pCR4-TOPO (TOPO TA Cloning Kit; Invitrogen). Following heat shock transformation of *Escherichia coli* TOPO10 (Invitrogen) cells with the ligation mixture, direct PCR on clones was performed using Taq Polymerase (Invitrogen) and the PCR products were purified with Sephadryl S-300 before sequencing.

TOPO10 clones, eight for each gene, were sequenced with gene-specific primers (Microsynth) (Table S1, see Supporting Information) using a BigDye terminator cycle-sequencing ready reaction kit (Applied Biosystems, Foster city, CA, USA). For removal of the unincorporated dye terminator, sequencing reaction products were purified by a DyeEx Kit (Qiagen, Hombrechtikon, Switzerland). Gene sequences were determined with a 3130 genetic analyser (Applied Biosystems) and compared using SeqMan software (DNA STAR Inc., Madison, WI, USA).

For Southern hybridization analysis, genomic DNA was isolated using a phenol–chloroform method (Borges *et al.*, 1990), and quantified spectrophotometrically (*A*₂₆₀/*A*₂₈₀). For each DNA

sample, 10 µg of genomic DNA (gDNA) was incubated overnight at 37 °C with 10 units of *Clal* restriction enzyme (New England Biolabs, Ipswich, MA, USA). Fully digested gDNA was separated on agarose gels and transferred to nylon membrane (Roche, Rotkreuz, Switzerland) as described previously (Sambrook *et al.*, 1989). A 1-kb *PiCesA3*-specific hybridization probe was obtained by PCR amplification using primers *PiCesA3fw* and *PiCesA3rev* (Table S1, see Supporting Information). Probe labelling was performed using an AlkPhos direct labelling kit with hybridization overnight at 55 °C, and two washing steps were performed following the manufacturer's protocol (GE Healthcare). The chemiluminescent signal was generated with CDP-Star (GE Healthcare) and detected using Kodak BioMax light film (Millian, Meyrin, Switzerland).

Genomic DNA for pyrosequencing was extracted from freeze-dried mycelium using the Qiagen DNeasy plant mini-kit. The extracted DNA was then used as a template for PCR employing the oligonucleotide primers *Pyr_Pinf_Ces3fwd* and *Pyr_Pinf_Ces3rev* (Table S1, see Supporting Information). The PCR conditions used an initial melt of 94 °C for 4 min, followed by 45 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 20 s, with a final extension step of 5 min at 72 °C. Biotinylated PCR products were then captured on streptavidin-coated beads (GE Healthcare), denatured (0.1 M NaOH) and washed using a Biotage (Uppsala, Sweden) vacuum prep workstation following the manufacturer's instructions. The pyrosequencing primer *Pyr_Pinf_Ces3seq* (Table S1, see Supporting Information) was then added to the biotinylated strand and the mixture was sequenced using Pyromark ID (Biotage). The gene copy number was assessed using Biotage allele quantification software. All determinations were repeated on six independent occasions, and statistical evaluation was made by calculation of the confidence interval.

For RT-PCR, total RNA was extracted using the Qiagen RNeasy plant mini-kit employing the protocol supplied by the manufacturer, with the addition of on-column DNase digestion. The synthesis of first-strand cDNA was carried out using a First Strand cDNA Synthesis kit (GE Healthcare, Amersham, UK) employing oligo-dT priming, following the protocol supplied by the manufacturer. For gene expression analysis, SYBR green real-time RT-PCR assays were carried out as described in Avrova *et al.* (2003). The *P. infestans* *PiactA* (actin A) gene was used as a constitutively expressed endogenous control and the relative expression of wild-type or mutant *PiCesA3* and transgenic *PiCesA3* G1105V was normalized against the expression levels in nonsporulating mycelium (assigned a relative expression value of 1.0), as described in Grenville-Briggs *et al.* (2008) and Judelson *et al.* (2008). Transgenic *PiCesA3* G1105V sequences were specifically targeted by the use of a reverse primer located within the 3' untranslated pTor vector sequence before the polyadenylation signal.

Transformation of *P. infestans*

The *PiCesA3* gene, encoding a cellulose synthase, was amplified from a plasmid clone of the gene from the MPD-insensitive mutant *Tmut3* using primers *CES3Fw* (incorporating a *Clal* site) and *CES3Rev* (incorporating a *NotI* site) (Table S1, see Supporting Information). Each 50 µL PCR contained the following components: 0.5 U Phusion Hot Start DNA polymerase (New England Biolabs), 10 µL of 5 × reaction buffer (New England BioLabs), 15 mM deoxynucleotide triphosphates (Promega, Southampton, UK), 30 µM forward and reverse primers (Table S1, see Supporting Information) and 10 ng *Tmut3 PiCesA3* plasmid DNA. Thermocycling conditions used an initial melt of 98 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 2 min. A final extension step of 72 °C for 10 min was included. PCR products were purified (Qiagen Minelute kit), digested with *Clal* and *NotI* restriction endonucleases (Promega) and purified again by Minelute. PCR products were directionally ligated, using T4 DNA ligase (Promega), into the *Clal* and *NotI* sites of vector pTor (Blanco and Judelson, 2005) for the constitutive overexpression from the *Bremia lactucae* *Ham34* promoter. Insert orientation and integrity were confirmed by DNA sequencing.

Stable transformation of *P. infestans* was achieved using a modified polyethylene glycol (PEG)–CaCl₂–lipofectin protocol (Judelson *et al.*, 1991). Modifications to this protocol were as described in Grouffaud *et al.* (2008).

ACKNOWLEDGEMENTS

We wish to thank Professor Paul Birch and Dr Dietrich Hermann for their support and encouragement. We would also like to thank Dr Mafalda Nina for fruitful discussions and helpful comments on the manuscript. SCW and AOA were supported by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Analysis of chromatograms from direct sequencing of the *PiCesA3* gene from strain 96, T30-4, Tmut3 and Tmut7. Strain 96

and T30-4 show a G residue at position 3417 and are homozygous. Tmut3 and Tmut7 show the mutated bases T and C, respectively, at position 3417.

Fig. S2 Southern blot analysis of genomic DNA from wild-type *Phytophthora infestans* (T30-4, 88069) and ethyl methane sulphonate (EMS) mutants (Tmut3, Tmut7) displaying mandipropamid (MPD) insensitivity. Genomic DNA was digested with *Clal* and hybridized to a 1-kb fragment specific to the *PiCesA3* gene. Molecular size markers (1 kb+, Qiagen) are indicated on the right.

Table S1 Oligonucleotide primers used for the sequencing of *Phytophthora infestans* genes encoding cell wall and PiCesA proteins, cloning of the *PiCesA3* gene, and reverse transcriptase-polymerase chain reaction (RT-PCR) of *PiCesA3*.

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