Genetic Analysis of Metalaxyl Insensitivity Loci in *Phytophthora infestans* Using Linked DNA Markers

Anna-Liisa Fabritius, Richard C. Shattock, and Howard S. Judelson

First and third authors: Department of Plant Pathology, University of California, Riverside 92521; and second author: School of Biological Sciences, University of Wales, Bangor, Gwynedd LL572UW, United Kingdom.

Accepted for publication 8 July 1997.

ABSTRACT

Fabritius, A.-L., Shattock, R. C., and Judelson, H. S. 1997. Genetic analysis of metalaxyl insensitivity loci in *Phytophthora infestans* using linked DNA markers. *Phytopathology* 87:1034-1040.

Previous studies indicated that incompletely dominant loci determine insensitivity by oomycetes to phenylamide fungicides such as metalaxyl. To compare the bases of insensitivity in different strains of the late blight pathogen, *Phytophthora infestans*, crosses were performed between sensitive isolates and isolates from Mexico, the Netherlands, and the United Kingdom that displayed varying levels of insensitivity. Segregation analyses indicated that metalaxyl insensitivity was determined primarily by one locus in each isolate, and that two of the isolates were heterozygous

and the other homozygous for the insensitive allele. Metalaxyl insensitivity was also affected by the segregation of additional loci of minor effect. DNA markers linked to insensitivity were obtained by bulked segregant analysis using random amplified polymorphic DNA (RAPD) markers and the Dutch and Mexican crosses. By studying the linkage relationships between these markers and the insensitivity in each cross by RAPD or restriction fragment length polymorphism analysis, it appeared that the same chromosomal locus conferred insensitivity in the Mexican and Dutch isolates. However, a gene at a different chromosomal position was responsible for insensitivity in the British isolate.

Additional keywords: fungicide resistance, quantitative trait.

The development of resistance to fungicides is a major problem in the management of plant diseases (36). Phenylamide fungicides such as metalaxyl, when they were first introduced, displayed excellent performance as systemic fungicides with protective, curative, and long-lasting activity against many important plant pathogenic fungi within the Peronosporales (38). However, strains insensitive to phenylamides have since appeared in many important crop systems (15). For example, not long after the introduction of metalaxyl to the European market in the 1970s, insensitive strains of the late blight pathogen *Phytophthora infestans* were detected (8,10) and, by the 1990s, insensitivity was reported in North America and other regions (9,17). Metalaxyl insensitivity has also developed in other *Phytophthora* species (1,16,21), downy mildews (3), and related pathogens (28,32,37), although phenylamides are still effective in many situations.

While the occurrence of resistant strains has been well documented, much remains to be learned concerning the genetic and molecular basis of resistance and the development of insensitive genotypes. Genetic analyses in *Albugo candida* (28), *Bremia lactucae* (5), *P. infestans* (39), *P. sojae* (1), and *Peronospora parasitica* (34) suggested that a semidominant locus determined insensitivity. Biochemical studies indicated that phenylamides inhibit the synthesis of ribosomal RNA, and showed that insensitivity in one isolate of *P. megasperma* and one of *P. infestans* were associated with decreased binding of metalaxyl to mycelial extracts (6,7,11). However, it remains to be determined if insensitivity in different isolates or species always has the same genetic or biochemical basis. Whether reduced sensitivity is due to a change in the fungicide target also remains to be demonstrated.

The objective of this study was to characterize the genetic basis of metalaxyl insensitivity in geographically diverse isolates of *P.*

infestans and to lay the groundwork for future molecular studies of the trait. This was achieved by studying the inheritance of resistance in crosses between insensitive strains, from Mexico, the Netherlands, and the United Kingdom, and sensitive isolates. The patterns of inheritance were further interpreted using DNA markers, obtained by bulked segregant analysis, that were linked to the insensitivity loci. The results indicated that some of the isolates were homozygous for the insensitive alleles of a semidominant gene of major effect, while others were heterozygotes. The linkage relationships between the DNA markers and the insensitivity genes suggested that a locus in a different chromosomal position determined insensitivity in the British isolate relative to the Dutch and Mexican isolates. Quantitative analyses of the progeny also indicated that their response to metalaxyl was significantly affected by minor or epistatic modifying genes, in addition to the major insensitivity locus.

MATERIALS AND METHODS

Strains. Isolates used in this study are listed in Table 1. These were obtained from infected potato or tomato plants or from P. Tooley (USDA-ARS, Fort Detrick, MD) or M. Coffey (University of California, Riverside). Experiments in the United States were performed under USDA-APHIS permits. Crosses were performed as described (24). Outcrossed progeny were distinguished from selfs or products of apomixis based on the inheritance of random amplified polymorphic DNA (RAPD) markers (43) from both parents. Isolates and progeny were maintained on rye agar (2) at 18°C.

Metalaxyl sensitivity assays. Responses to fungicide were determined by growing strains on rye agar plates containing 0, 0.5, and 5.0 µg/ml (active ingredient) of metalaxyl; several previous studies have supported the correlation between this in vitro assay and in planta tests (17). The assay was performed by placing two 5 × 5-mm agar plugs on opposite sites of a 100-mm-diameter plate that was incubated in the dark at 18°C. At 3, 6, 9, and 12 days after inoculation, the radius of each colony was recorded using two

Corresponding author: H. S. Judelson; E-mail address: judelson@ucr.ac1.ucr.edu

Publication no. P-1997-0814-01R

© 1997 The American Phytopathological Society

measurements taken at a 90° angle. The relative insensitivity of each strain was determined by calculating the ratio of mean radial growth on the metalaxyl-amended media versus that on media without fungicide. Data from 9- or 12-day measurements are reported in the Results section, although the earlier time points were often useful to help confirm trends. Statistical analyses were performed using the Statview 4.02 program for Macintosh (Abacus Concepts Inc., Berkeley, CA).

Polymerase chain reaction (PCR) analyses. DNA for PCR assays was extracted as described (23). RAPD markers linked to metalaxyl insensitivity were obtained by bulked segregant analysis (30) using random 10-nucleotide (nt) primers (Operon Technologies Inc., Alameda, CA) and bulks of seven to eight progeny each. Reaction products were separated by electrophoresis in Tris-borate-EDTA (TBE: 89 mM Tris, 89 mM H₃BO₃, and 2 mM Na₂ EDTA) gels containing 0.5% agarose and 0.55% Infinity Agarose Enhancer (Oncor Inc., Gaithersburg, MD) at 5 V/cm and visualized by staining with ethidium bromide. Statistical analyses of linkages were performed using the MapManager program (27).

Hybridization studies. Restriction fragment length polymorphism (RFLP) analyses were performed as described using ³²P-labeled probes (24). Probes linked to metalaxyl insensitivity were derived from RAPD bands that had been cloned into pCRII (Invitrogen Corp., San Diego, CA). Only the RAPD band representing locus *W4* could be used as an intact probe for RFLP studies, as the others contained repetitive DNA. For locus *AL14*, a 450-base pair *EcoRI-HindIII* subfragment lacking repetitive DNA was identified that was used as the hybridization probe. Probes for actin (a gift of S. Unkles) or mating locus-linked marker *B1* were used as controls (22). Densitometric comparisons of hybridization signals were performed using a Hewlett-Packard ScanJet 3C scanning device (Hewlett-Packard Co., Palo Alto, CA) interfaced to a Macintosh 7100/66 computer (Apple Computer, Inc., Cupertino, CA) and the NIH Image program (W. Rasband, National Institutes of Health, Bethesda, MD).

RESULTS

Populations segregating for insensitivity. Three isolates exhibiting low sensitivity to metalaxyl were selected for analysis based on their genotypic, geographic, and phenotypic diversity (Table 1). British isolate 88-1-1 was highly insensitive to both 0.5 and 5 µg/ml of metalaxyl, while isolate 618 from central Mexico and isolate 1114 from the Netherlands were similarly insensitive to 0.5 µg/ml. Different dose responses were displayed by 1114 and 618 based on assays at 5 µg/ml, however. Analyses of isozyme, RAPD, RFLP, and avirulence loci suggested that these strains were not closely related (data not shown). Crosses were established between these insensitive strains and sensitive strains to obtain populations for studying the genetic basis of the phenotype and for DNA mapping.

The analysis of crosses derived from isolate 88-1-1 suggested that the strain was homozygous for a semidominant locus determining the majority of the insensitive phenotype. Most F₁ progeny of 88-1-1 and sensitive isolate E13a displayed levels of metalaxyl insensitivity intermediate to that of the parents, as measured by the ratio of radial growth on media amended with 0.5 µg/ml of metalaxyl to that on media without fungicide (Fig. 1). Crosses between F₁ offspring and sensitive strains yielded similar numbers of relatively sensitive and insensitive types. For example, a cross between an F₁ offspring of 88-1-1 and E13a (progeny 2.16, which displayed 55 ± 4% relative growth in 0.5 µg/ml of metalaxyl) and sensitive isolate Ca65 yielded 10 and 11 relatively sensitive and insensitive progeny, respectively. Similar results were obtained from crosses between other F₁ strains and sensitive isolates, which yielded 13 relatively sensitive and eight relatively insensitive progeny (data not shown). These data were consistent with a 1:1 segregation pattern indicative of heterozygosity at a major locus ($\chi^2 = 0.38$, $P = 0.55$).

A complication in this analysis was that placing progeny into discrete classes was difficult, and this was probably due to the segregation of additional modifying loci. For example, the relative resistance scores of 88-1-1 × E13a offspring ranged from 40 to 100% (Fig. 1). Broad ranges of resistance scores were also present within the insensitive and sensitive classes of Ca65 × 2.16 offspring (49 to 98% and 1 to 26%, respectively), and several offspring were more insensitive than 2.16. Large ranges were observed in tests at both 0.5 and 5 µg/ml of metalaxyl (data not shown). In assays repeated several times over 6 months, consistent rankings were obtained for strains selected from the high, middle, and low ends of each of the relatively insensitive and sensitive classes of Ca65 × 2.16 progeny. Similarly, consistency in rankings was observed in repeated assays of several of the 88-1-1 × E13a offspring that showed higher, average, and lower levels of insensitivity. Therefore, the distributions of resistance scores could not be attributed to error or week-to-week strain variation. Instead, the data were consistent with the hypothesis that insensitivity was determined by the combined action of one major gene plus several loci of lesser effect. Another factor that might contribute to the distribution of scores could be differences in the dosage of the major gene due to aneuploidy, although this was unsupported by later experiments.

Data from crosses involving 618 or 1114 and sensitive isolates were consistent with the former isolates being heterozygous at a major insensitivity locus and containing additional modifying loci. Nineteen of the 1114 × 510 progeny were classified as relatively insensitive and 23 were relatively sensitive, and 19 of the 1306 × 618 progeny were relatively insensitive and 16 were relatively sensitive (illustrated for representative progeny in Fig. 1). The points of division between these classes were consistent with the data and fit well with a 1:1 segregation ratio indicative of heterozygosity of the insensitive parents ($\chi^2 = 0.38$, $P = 0.55$ and $\chi^2 = 0.26$, $P = 0.62$ for the 1114 × 510 and 1306 × 618 crosses, respectively). As in the 88-1-1 lineage, other loci also appeared to segregate, which resulted in a broad range of values within each class. However, the same groupings were suggested by analyses at 0.5 and 5 µg/ml of metalaxyl, and subsequent mapping studies using molecular markers supported the groupings.

As an earlier study reported reduced fitness of insensitive individuals (41), the possibility of a relationship between growth rate in the absence of metalaxyl and response to the fungicide was tested. Weak negative correlations between growth and insensitivity were observed in each of the progeny of 2.16, 1114, and 618 (correlation coefficients of -0.31, -0.29, and -0.15, with P values of 0.06, 0.07, and 0.3, respectively).

DNA markers linked to the major insensitivity gene. Screens for DNA markers linked to the locus determining the majority of insensitivity were executed for each segregating population using RAPD primers and bulked segregant analysis. For crosses 1114 × 510, 1306 × 618, and Ca65 × 2.16, approximately 1,000 random 10-nt primers were used to amplify bands from a pool (bulk) of

TABLE 1. Characteristics of strains of *Phytophthora infestans* used in this study

Isolate	Source	Mating type	Relative growth in presence of metalaxyl (%) ^a	
			0.5:0	5:0
88-1-1	United Kingdom, 1988	A1	97 ± 3	83 ± 4
1114	The Netherlands, 1985	A1	81 ± 11	52 ± 15
618	Central Mexico, 1987	A2	84 ± 7	29 ± 6
2.16	(88-1-1 × E13a) F ₁	A2	55 ± 4	35 ± 10
Ca65	California, United States, 1980	A1	4 ± 3	2 ± 2
E13a	Egypt, 1984	A2	11 ± 9	2 ± 2
510	Central Mexico, 1983	A2	6 ± 2	6 ± 2
1306	California, United States, 1982	A1	8 ± 3	7 ± 2

^a Relative growth was determined as the percent radial growth observed on rye agar containing 0.5 or 5 µg/ml of metalaxyl divided by the amount of growth on media lacking metalaxyl. Indicated is the average result and standard deviation from at least three replicates.

template DNA from six to eight sensitive progeny and from DNA pooled from an equal number of insensitive progeny. These pools were constructed using individuals of extreme phenotypes to focus on the major insensitivity locus, as done in previous studies involving quantitative trait loci (31). Polymorphisms between the bulks were validated by tests of individual progeny, with an acceptance criteria of log of the likelihood ratio (LOD) ≥ 3.0 (35).

These assays identified three DNA loci linked to the major insensitivity gene(s) (Table 2). Two were linked to the insensitivity locus of 1114 in *cis* (i.e., transmitted from insensitive parent to insensitive progeny) and, based on the principle of minimizing recombinants, these mapped to the same side of the insensitivity locus. The segregation of the RAPD band representing locus W4

is illustrated in Figure 2A for selected 1114 $\times$ 510 progeny. The third marker was linked to the resistance locus of 618 and was also in *cis* to the insensitive allele. RAPD bands significantly linked to the metalaxyl insensitivity locus were not identified in the Ca65 $\times$ 2.16 screen, possibly due to a lower rate of polymorphism or the presence of recombinants within the progeny pools (bulks) used in the assay.

The validity of these markers rested upon the assumption that metalaxyl insensitivity was determined largely by a single locus. In light of the semiquantitative nature of the insensitivity trait, this assumption was statistically assessed using Student's *t* test to analyze marker genotype versus insensitivity phenotype. This supported the distinct nature of the classes identified by the DNA loci

Fig. 1. Response to metalaxyl in parents and progeny of crosses of *Phytophthora infestans*. The relative resistance of parents and progeny of crosses 88-1-1 $\times$ E13a, Ca65 $\times$ (88-1-1 $\times$ E13a) F₁ 2.16, 1114 $\times$ 510, and 1306 $\times$ 6636 (A1 $\times$ A2) are presented as the percent growth on media amended with 0.5 μ g/ml of metalaxyl relative to that on unamended media. Bars are labeled as denoting the parents (Pa, with A1 and A2 parents drawn from left to right) or representative progeny (S or I, corresponding to sensitive or insensitive classifications used to calculate linkage with DNA markers). Indicated beneath each bar is the W4 genotype for the indicated strain based on random amplified polymorphic DNA analysis (1114 $\times$ 510) or restriction fragment length polymorphism analysis (Ca65 $\times$ [881-1 $\times$ E13a] F₁ and 1306 $\times$ 6636), with ■ and □ representing alternate alleles. Error bars represent range of values obtained from replicated assays, with the progeny within each cross assayed on the same day using the same batch of medium. Only representative progeny are shown for the crosses, because some progeny were assayed on separate days; total progeny numbers are presented in the text.

(*W4* versus insensitivity in 1114×510 , $t_{\text{obs}} = 4.84$, 24 degrees of freedom [df], and P of homogeneity $\approx 10^{-4}$; and *AI18* versus insensitivity in 1306×618 , $t_{\text{obs}} = 4.72$, 22 df, and $P \approx 10^{-4}$).

Comparative mapping of insensitivity in 1114 and 618. To establish if the same locus was the primary determinant of insensitivity in isolates 618 and 1114, the linkage of the DNA and fungicide response loci in the different crosses was tested. It was necessary to do this using RFLP methods, since the RAPD patterns were not conserved in the different parents. Consequently, the original RAPD bands were cloned into plasmids and used as probes to detect and map polymorphisms in the crosses. In some cases, the entire inserts were unsuited for RFLP analysis, since they contained repetitive DNA sequences and, in such cases, subfragments of the bands were tested for single-copy sequences. Using such methods, suitable single-copy RFLP probes were obtained for loci *W4* and *AL14*.

Since these two loci had been already characterized in isolate 1114, the probes were used to study the 1306×618 population. A *Bgl*III polymorphism between those parents was used to demonstrate that the major insensitivity locus in isolate 618 was also linked to *W4*, with three recombinants identified out of 24 offspring (illustrated for representative progeny in Figs. 1 and 2B). This was consistent with insensitivity in isolates 1114 and 618 being determined by the same chromosomal region and, possibly, by the same gene. Locus *AL14* could not be mapped in this cross, since it was monomorphic in each of 40 restriction digests tested. Nevertheless, by mapping *W4* in the 1306×618 cross and combining the results with those for *W4* and *AI18* in the 1114×510 cross, it was possible to orient all of the loci and construct a consensus genetic map (Fig. 3). In this map, the fungicide resistance locus has been named *MEX* to correspond to terminology suggested in earlier studies (1,40).

The genetic basis of fungicide response in 1114 and 618 was tested further by crossing these isolates and scoring the inheritance of the DNA loci and *MEX*. This would indicate if *MEX* was semi-dominant, which could not be assumed, and would confirm that the same locus was involved in the two isolates; if both contentions were true, then 1114×618 progeny should include highly resistant, intermediate, and sensitive types. This was consistent with the results, although divisions between the classes were blurred, presumably due to the minor loci (Fig. 4). More informative data were obtained by comparing the inheritance of insensitivity and RAPD or RFLP alleles of *W4* present in *cis* with the insensitive *MEX* alleles. Those results indicated that, in most cases, the highly insensitive offspring were homozygous for the insensitive alleles, intermediate types were heterozygous, and sensitive offspring were homozygous for sensitivity (coded in lower part of Fig. 4). Due to recombination, the DNA genotypes and phenotypes did not absolutely coincide; for example, 11 of the 13 most insensitive offspring were homozygous for the resistance-linked alleles of *W4*, which was consistent with the genetic distances indicated in Table 2. Although a 1:2:1 ratio of resistant homozygotes, heterozygotes, and sensitive homozygotes was expected, the heterozygote class appeared underrepresented both phenotypically and for the DNA

markers, in which a 13:10:14 ratio was observed. This could be accounted for by distorted segregation, as evidenced by the occurrence of the two heterozygote genotypes in a 10:1 rather than the normal 1:1 ratio.

Besides enabling the comparative mapping of the major insensitivity loci, the DNA markers were also useful in suggesting whether insensitivity had evolved independently in 1114 and 618. Several RFLPs between 1114 and 618 were observed using probes for *W4*, which suggested the independent lineage of that chromosomal region and the independent development of insensitivity. The possibility that the polymorphisms were introgressed into a common insensitive lineage cannot be excluded, however.

The DNA markers also enabled tests of whether the extreme insensitive offspring were aneuploid, possibly containing an extra resistant *MEX* allele. This was considered, since nondisjunction has been reported in *Phytophthora* (12,13,22) and, therefore, the most insensitive strains might represent $2N+1$ aneuploids. However, this

Fig. 2. Segregation of *W4* alleles in crosses of *Phytophthora infestans*. **A**, Inheritance of 0.6-kb band obtained using random amplified polymorphic DNA primer *W4*. Indicated are the amplification products for parents (Pa) and six representative insensitive (I) and six representative sensitive (S) progeny. Insensitive and sensitive parents were 1114 (lane 1) and 510 (lane 2), respectively. **B**, Inheritance of 5.9-kb restriction fragment length polymorphism (RFLP) band detected in *Bgl*III digests using a probe for *W4* in sensitive isolate 1306 (lane 1), insensitive isolate 618 (lane 2), and their representative progeny. **C**, Inheritance of 5.9-kb RFLP band detecting *Bgl*III alleles of *W4* in sensitive isolate Ca65 (lane 1), insensitive strain 2.16 (F_1 progeny of 88-1-1 and E13a, lane 2), and their representative progeny. Molecular weights were determined using 123-bp ladders or *Pst*I digests of bacteriophage λ .

TABLE 2. Random amplified polymorphic DNA markers linked to determinants of metalaxyl insensitivity in isolates of *Phytophthora infestans*

Cross ^a	Primer ^b	Band size in kb	Recombination fraction and LOD score ^c	Phase with resistance allele
1114 $\times$ 510	W4	0.60	0.10 (6.9)	<i>cis</i>
1114 $\times$ 510	AL14	1.46	0.14 (5.8)	<i>cis</i>
1306 $\times$ 618	AI18	1.21	0.14 (3.5)	<i>cis</i>

^a The cross in which the polymorphism was detected, with the band originating in the relatively insensitive parent (1114 or 618).

^b Standard primers from Operon Technologies Inc., Alameda, CA.

^c Calculated from the indicated cross. The log of the likelihood ratio (LOD) score (35) is shown in parentheses.

Fig. 3. Consensus map of DNA markers flanking the major locus conferring metalaxyl insensitivity (*MEX*) in isolates 1114 and 618 of *Phytophthora infestans*. Indicated are the DNA markers and their mean linkage to *MEX*, calculated as recombination fraction, as determined by random amplified polymorphic DNA and restriction fragment length polymorphism analyses.

possibility was not supported by densitometric comparisons of the hybridization signals obtained with probes for *W4* and *AL14* and with probes for actin and mating type loci; the ratio of the hybridization signals for *W4* versus actin in a *BclI* digest, for example, were essentially the same for the individuals of extreme and average phenotypes ($\pm 12\%$, within the level of experimental error). Therefore, the extreme phenotypes are more likely attributable to the assortment of the minor loci.

Comparative mapping of insensitivity in 88-1-1. In contrast to the findings with 1114 and 618, a different region of the genome appeared to determine insensitivity in 88-1-1 based on RFLP analyses of $\text{Ca65} \times (88-1-1 \times \text{E13a})$ F_1 crosses. As illustrated for representative progeny in Figures 1 and 2C, *W4* appeared unlinked to the insensitivity determinant from 88-1-1 (calculated at 0.43 cM based on 42 $\text{Ca65} \times [88-1-1 \times \text{E13a}]$ F_1 progeny). Locus *AL14* also appeared unlinked to the metalaxyl response locus in 88-1-1, while maintaining its linkage to *W4* at a recombination fraction of 0.00. The possibility was considered that a higher rate of recombination existed within the progeny of 88-1-1, which made *W4* and *MEX* appear unlinked. However, this appeared unlikely due to the conserved linkage of *W4* and *AL14*. Morton's *M* test (33) also strongly supported the determination of insensitivity by different loci in isolates 1114 and 618 versus 88-1-1 ($P = 0.97$ for genetic heterogeneity). Also tested was whether the level of insensitivity displayed by the $\text{Ca65} \times (88-1-1 \times \text{E13a})$ F_1 progeny in the two *W4* genotypic classes was significantly different; this might have been the case if the same *MEX* locus as in 1114 and 618 was involved, but the modifying or epistatic loci led to misclassification of some progeny. However, *t* test analysis did not support a significant difference between those groups ($t_{\text{obs}} = 0.68$, 40 df, and $P \approx 0.5$).

DISCUSSION

In this study, the basis of insensitivity to metalaxyl in three diverse isolates of *P. infestans* was examined. Each isolate contained a semidominant locus that was responsible for a majority of the phenotype, but a significant role was also played by additional loci of minor effect that resembled quantitative trait loci. This compares with previous studies in several oomycetes in which the determi-

nation of phenylamide insensitivity by major genes alone was emphasized (1,3,28,34,39), although some studies did note anomalies that might have been explained by the involvement of multiple genes (20). Two of the three isolates proved to be heterozygous for the insensitive allele of the major locus, in contrast to previous studies that identified only homozygosity in natural isolates of several oomycetes (1,3,28,34,39). DNA markers were identified that flanked the major insensitivity locus, and their segregation in crosses was consistent with the existence of heterogeneous mechanisms for conferring insensitivity in *P. infestans*.

With other fungicides, there are precedents for the determination of resistance by both major and minor genes. Resistance is usually considered to be regulated by major genes as with the benzimidazoles, or to be under polygenic control as with the sterol demethylation inhibitors (14). However, there are cases in which both types of loci are involved as with triazole resistance in *Nectria haematococca* (25). In *P. infestans*, the additive effect of the minor loci on metalaxyl insensitivity can be substantial, accounting for as much as 50% of the resistance level in the *MEX* heterozygotes described in this study. It follows that matings in natural populations may result in the development of strains with significantly increased levels of fungicide insensitivity. That such minor genes are present in natural populations of *P. infestans* is consistent with reports that both "sensitive" and "resistant" isolates display substantial quantitative variation in their response to metalaxyl (17,29), and similar observations have been made in other species (21). Although some of this variation might be due to allelic differences at *MEX*, this study suggests that variability at the minor loci may be primarily responsible.

While the genetics of phenylamide insensitivity has been addressed in a few oomycete species (1,3,28,34,39), interisolate comparisons have not been reported. The interpretation of such analyses would be difficult based on phenotype alone. This is due to the complexity of deciphering interactions involving unknown interallelic or interlocus effects. For example, in the case of two hypothetical major genes such as *MEX1* and *MEX2*, it could not be predicted whether the *MEX1MEX2/mex1mex2* double heterozygote would display a level of insensitivity greater, equal, or less than that of a *MEX1mex2/MEX1mex2* strain. Moreover, the minor

Fig. 4. Segregation of insensitivity phenotype and *W4* alleles in a cross between isolates 1114 and 618 of *Phytophthora infestans*. The relative insensitivity levels of parents (black bars) and representative progeny (grey bars) are presented as the percent growth on media amended with 5 $\mu\text{g/ml}$ of metalaxyl relative to that on nonamended media. Error bars represent range of values from replicated assays. Alleles of *W4* inherited from the parents are denoted at the base of the figure as W4-1114 or W4-618 (representing alleles from 1114 scored by random amplified polymorphic DNA analysis or from 618 as identified by restriction fragment length polymorphism analyses using *BstEII* digests, respectively), with closed squares representing the allele in *cis* with the insensitive *MEX* allele.

loci would confound the grouping of progeny into clear phenotypic classes and make the formulation of genetic models difficult. Due to these complications, the DNA markers employed here were valuable, since they enabled both genotypic and phenotypic analyses, although the DNA markers might be associated with uncertainties as well. For example, the absence of linkage between the markers from 1114 and insensitivity in 88-1-1 might reflect rearrangements in chromosomes between the isolates instead of the occurrence of different major insensitivity loci. In the limited analyses performed to date, there are precedents for translocations within *P. infestans*, although most markers appear well conserved (22).

The nature of the molecular changes that confer insensitivity to phenylamides currently can only be the subject of speculation. Phenylamides are reported to specifically inhibit the synthesis of ribosomal RNA, but their precise target is unknown (6,7,11). RNA polymerase I is sometimes suggested as the target, but the complex nature of the eukaryotic transcriptional apparatus presents multiple potential targets (19). Moreover, the ability of phenylamides to impair transcription in mycelial extracts, but not purified nuclei, may suggest a less direct mode of action (7). Phenylamides were reported to bind to extracts of sensitive, but not resistant, isolates (6), implying that a change in the target may cause insensitivity; but that may not hold for all isolates, particularly in light of the heterogeneity suggested by our results. Assuming that more than one major locus may be implicated in *P. infestans*, the genetics of phenylamide insensitivity would be similar to that of carboximide and kasugamycin resistance in which multiple mechanisms have been described (18,42). Considering that genes of related function are often clustered in eukaryotes (4,26), it is even possible that insensitivity in 1114 and 618 is determined by distinct, albeit tightly linked, genes.

DNA markers linked to metalaxyl insensitivity loci have applications beyond those presented here. Such markers may be used to study the development and spread of insensitivity through populations or for assays to predict insensitivity; probes tightly linked to the gene should be more appropriate than the unlinked markers that are currently employed (17). Also, whether homozygous resistant isolates resulted from selfing, mitotic crossing-over, or second mutations in heterozygotes might be determined with the aid of the flanking DNA markers. Such studies may indicate how resistance to new fungicides might develop. These applications assume that loci such as *W4* will be linked to insensitivity in the targeted population, which will not necessarily be true as observed with isolate 88-1-1. Studies of other populations, such as those in the United States, will help indicate the diversity of resistance mechanisms within *P. infestans*. Through a positional cloning strategy, the markers also provide the means to isolate the gene itself, which will be best suited to the above uses. The gene may also be useful as a selectable marker for DNA-mediated transformation, particularly for obligate pathogens such as the downy mildew fungi in which in planta selection schemes are required. The analysis of the gene may also reveal the actual target of the phenylamides.

ACKNOWLEDGMENTS

We thank Ciba-Geigy for providing metalaxyl and other assistance. We also thank M. Coffey and P. Tooley for supplying isolates, and J. Griffith and S. Roberts for technical assistance.

LITERATURE CITED

- Bhat, R. G., McBlain, B. A., and Schmitthenner, A. F. 1993. The inheritance of resistance to metalaxyl and to fluorophenylamine in matings of homothallic *Phytophthora sojae*. Mycol. Res. 97:865-870.
- Caten, C. E., and Jinks, J. L. 1968. Spontaneous variability of isolates of *Phytophthora infestans*. I. Cultural variation. Can. J. Bot. 46:329-348.
- Crute, I. R. 1987. The occurrence, characteristics, distribution, genetics, and control of a metalaxyl-resistant pathotype of *Bremia lactucae* in the United Kingdom. Plant Dis. 71:763-767.
- Crute, I. R. 1994. Gene-for-gene recognition in plant-pathogen interactions. Philos. Trans. R. Soc. Lond. B Biol. Sci. 346:345-349.
- Crute, I. R., and Harrison, J. M. 1988. Studies on the inheritance of resistance to metalaxyl in *Bremia lactucae* and on the stability and fitness of field isolates. Plant Pathol. 37:231-250.
- Davidse, L. C. 1995. Phenylamide fungicides—Biochemical action and resistance. Pages 347-354 in: Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. H. Lyr, ed. Gustav Fischer Verlag, New York.
- Davidse, L. C., Hofman, A. E., and Velthuis, G. C. M. 1983. Specific interference of metalaxyl with endogenous RNA polymerase activity in isolated nuclei from *Phytophthora megasperma* f. sp. *medicaginis*. Exp. Mycol. 7:344-361.
- Davidse, L. C., Looijeu, D., Turkensteen, L. J., and van der Als, D. 1981. Occurrence of metalaxyl-resistant strains of potato blight in Dutch potato fields. Neth. J. Plant Pathol. 87:65-68.
- Deahl, K. L., Inglis, D. A., and DeMuth, S. P. 1993. Testing for resistance to metalaxyl in *Phytophthora infestans* isolates from northwestern Washington. Am. Potato J. 70:779-795.
- Dowley, L. J., and O'Sullivan, E. 1981. Metalaxyl-resistant strains of *Phytophthora infestans* (Mont.) de Bary in Ireland. Potato Res. 24:417-421.
- Fisher, D. J., and Hayes, A. L. 1984. Studies of mechanisms of metalaxyl fungitoxicity and resistance to metalaxyl. Crop Prot. 3:117-185.
- Förster, H., and Coffey, M. D. 1990. Mating behavior of *Phytophthora parasitica*—Evidence for sexual recombination in oospores using DNA restriction fragment length polymorphisms as genetic markers. Exp. Mycol. 14:351-359.
- Fyfe, A. M., and Shaw, D. S. 1992. An analysis of self-fertility in field isolates of *Phytophthora infestans*. Mycol. Res. 96:390-394.
- Georgopoulos, S. G. 1995. The genetics of fungicide resistance. Pages 39-51 in: Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. H. Lyr, ed. Gustav Fischer Verlag, New York.
- Gisi, U., and Cohen, Y. 1996. Resistance to phenylamide fungicides: A case study with *Phytophthora infestans* involving mating type and race structure. Annu. Rev. Phytopathol. 34:549-572.
- Goodwin, S. B., and McGrath, M. T. 1995. Insensitivity to metalaxyl among isolates of *Phytophthora erythroseptica* causing pink rot of potato in New York. Plant Dis. 79:967.
- Goodwin, S. B., Sujkowski, L. S., and Fry, W. E. 1996. Widespread distribution and probable origin of resistance to metalaxyl in clonal genotypes of *Phytophthora infestans* in the United States and western Canada. Phytopathology 86:793-800.
- Gunatillake, I. A. U. N., Arst, H. N., and Scazzocchio, C. 1976. Three genes determine the carboxin sensitivity of mitochondrial succinate oxidation in *Aspergillus nidulans*. Genet. Res. 26:297-305.
- Halle, J.-P., and Meisterernst, M. 1996. Gene expression: Increasing evidence for a transcriptosome. Trends Genet. 12:161-163.
- Harrison, B. 1991. The genetics of *Phytophthora infestans*. Ph.D. thesis. University of Wales, Bangor.
- Hunger, R. M., Hamm, P. B., Horner, C. E., and Hansen, E. M. 1982. Tolerance of *Phytophthora megasperma* isolates to metalaxyl. Plant Dis. 66:645-649.
- Judelson, H. S. 1996. Genetic and physical variability at the mating type locus of the oomycete, *Phytophthora infestans*. Genetics 144:1005-1013.
- Judelson, H. S. 1996. Chromosomal heteromorphism linked to the mating type locus of the oomycete *Phytophthora infestans*. Mol. Gen. Genet. 252:155-161.
- Judelson, H. S., Spielman, L. J., and Shattock, R. C. 1995. Genetic mapping and non-Mendelian segregation of mating type loci in the oomycete, *Phytophthora infestans*. Genetics 141:503-512.
- Kalamarakis, A. E., Demopoulos, V. P., Ziogas, B. N., and Georgopoulos, S. G. 1989. A highly mutable major gene for triadimenol resistance in *Nectria haematococca* var. *cucurbitae*. Neth. J. Plant Pathol. 95(suppl. 1):109-120.
- Keller, N. P., and Hohn, T. M. 1997. Metabolic pathway gene clusters in filamentous fungi. Fungal Genet. Biol. 21:17-29.
- Manly, K. F. 1993. A Macintosh program for storage and analysis of experimental genetic mapping data. Mamm. Genome 4:303-313.
- Mathur, S., Wu, C., Liu, J. Q., and Rimmer, S. R. 1995. Inheritance of virulence and fungicide resistance in *Albugo candida*. Can. J. Plant Pathol. 17:360.
- Matuszak, J. M., Fernandez-Elquezabal, J., Gu, W. K., Villarreal-Gonzalez, M., and Fry, W. E. 1994. Sensitivity of *Phytophthora infestans* populations to metalaxyl in Mexico: Distribution and dynamics. Plant Dis. 78:911-916.
- Micheltore, R. W., Paran, I., and Kesseli, R. V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions using segregating polymorphisms. Proc. Natl. Acad. Sci. U.S.A. 88:9828-9832.

31. Miklas, P. N., Johnson, E., Stone, V., Beaver, J. S., Montoya, C., and Zapata, M. 1996. Selective mapping of QTL conditioning disease resistance in common bean. *Crop Sci.* 36:1344-1351.
32. Morton, H. V., and Urech, P. A. 1988. History of the development of resistance to phenylamide fungicides. Pages 59-62 in: *Fungicide Resistance in North America*. C. J. Delp, ed. The American Phytopathological Society, St. Paul, MN.
33. Morton, N. E. 1956. The detection and estimation of linkage between the genes for elliptocytosis and the Rh blood type. *Am. J. Hum. Genet.* 8:80-96.
34. Moss, N. A., Crute, I. R., and Lucas, J. A. 1994. Laboratory production of oospores of *Peronospora parasitica* (crucifer downy mildew) and the recovery and characterization of sexual progeny from crosses between isolates with different host specificity. *Plant Pathol.* 43:713-725.
35. Ott, J. 1985. *Analysis of Human Genetic Linkage*. Johns Hopkins University Press, Baltimore.
36. Russell, P. E. 1995. Fungicide resistance: Occurrence and management. *J. Agric. Sci.* 124:317-323.
37. Sanders, P. L. 1984. Failure of metalaxyl to control Pythium blight on turfgrass in Pennsylvania. *Plant Dis.* 68:776-777.
38. Schwinn, F., and Staub, T. 1995. Oomycete fungicides. Pages 323-346 in: *Modern Selective Fungicides, Properties, Applications, Mechanisms of Action*. H. Lyr, ed. Gustav Fischer Verlag, New York.
39. Shattock, R. C. 1988. Studies on the inheritance of resistance to metalaxyl in *Phytophthora infestans*. *Plant Pathol.* 37:4-11.
40. Shaw, D. S. 1991. Genetics. Pages 131-170 in: *Phytophthora infestans, the Cause of Late Blight of Potato*. Advances in Plant Pathology, vol. 7. D. S. Ingram and P. H. Williams, eds. Academic Press, San Diego, CA.
41. Staub, T., Dahmen, H., Urech, P., and Schwinn, F. 1979. Failure to select for in vivo resistance in *Phytophthora infestans* to acylalanine fungicides. *Plant Dis. Rep.* 63:385-389.
42. Taga, M., Nakagawa, H., Tsuda, M., and Ueyama, A. 1978. Ascospore analysis of kasugamycin resistance in the perfect stage of *Pyricularia oryzae*. *Phytopathology* 68:815-817.
43. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1991. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6536.