Multiple Loci Determining Insensitivity to Phenylamide Fungicides in *Phytophthora infestans*

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ABSTRACT

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The diversity of mechanisms causing insensitivity to phenylamide fungicides in *Phytophthora infestans* was addressed by comparative genetic analyses of isolates from North America, Europe, and Mexico. Both semi-dominant major loci (*MEX* loci) and genes of minor effect were previously shown to determine insensitivity based on studies of isolates from Europe and Mexico. In this investigation, genetic analyses of three highly insensitive isolates from the United States and Canada revealed a similar pattern involving major and minor loci. However, *MEX* alleles in two Ca-

nadian isolates conferred higher levels of insensitivity than those examined previously, particularly in a heterozygous state. This suggested that not all *MEX* alleles in *P. infestans* were functionally equivalent. The chromosomal locations of the major insensitivity loci were also shown to vary in different isolates based on linkage analyses performed with the aid of DNA markers. The major determinant of insensitivity in the North American, Dutch, and Mexican isolates mapped to the same locus, which was named *MEX1*. In a British isolate, a different locus, dubbed *MEX2*, was implicated that mapped to the same linkage group as *MEX1* but to a distinct site.

Additional keywords: late blight, metalaxyl, quantitative trait.

Late blight has reemerged over recent years as a significant threat to potato and tomato production worldwide (12). This can be largely attributed to genetic changes in the pathogen, *Phytophthora infestans* (Mont.) de Bary. The development of insensitivity to phenylamide fungicides such as metalaxyl has been a particular problem (14). When first introduced, phenylamides displayed good protective and curative systemic activities against a broad range of oomyceteous plant pathogens (35). However, the effectiveness of these compounds for controlling late blight was challenged by the appearance of insensitive isolates of *P. infestans* in Europe, North America, and other regions over the past 2 decades (4,7,8,16,28,30,31). Insensitive strains have also been reported for several other species of *Phytophthora* (1) and for downy mildews (6,31,32), white rusts (27), and *Pythium* (34).

Much remains to be learned concerning the genetic and molecular bases of insensitivity and the processes through which that trait evolved. Insensitive strains of *P. infestans* from different parts of the world are genetically diverse based on their isozyme and DNA fingerprints (11,16), and it follows that insensitivity likely evolved on multiple occasions. However, it is not known whether a change at the same locus is always responsible. By analogy with other systems, insensitivity might result from a variety of mechanisms including inactivation, altered transport, or altered metabolism of the fungicide, or a change at the phenylamide target (39). Therefore, several different loci could contribute to insensitivity. The precise cellular target of the phenylamides remains to be elucidated, but biochemical studies indicated an effect on the synthesis of ribosomal RNA (7,10). Understanding the basis of insensitivity would help illuminate the target and metabolism of this important class of fungicides.

A previous study by our laboratory revealed a complex pattern of inheritance for insensitivity in *P. infestans* involving both major

and minor loci (9). That major determinants (*MEX* loci) existed concurred with conclusions by other groups based on studies of various *Phytophthora* spp. (1,3,36,40). However, our investigation indicated the presence of additional loci that exerted minor effects or were epistatic to *MEX* as in a quantitative trait (9), and this was subsequently supported by others (25). Genetic analyses of insensitive strains of British, Dutch, and Mexican origins showed that those that were highly insensitive were homozygous for the insensitive *MEX* allele, while intermediately insensitive strains were heterozygotes. This indicated that the *MEX* alleles present in those isolates were semidominant (9,36). The existence of two distinct *MEX* loci was proposed since a DNA marker linked to insensitivity in Dutch and Mexican isolates assorted independently of that phenotype in a British strain. However, this claim was made tentatively, because flanking markers could not be tested and, consequently, some alternative models could not be excluded. For example, a higher rate of recombination near *MEX* in the British isolate could have obscured its linkage with the DNA marker.

The current study had two main objectives aimed at expanding our understanding of the inheritance and evolution of phenylamide insensitivity in *P. infestans*. First, analyses were expanded to include strains from North America. Second, whether more than one major *MEX* locus existed in *P. infestans* was addressed with greater precision through the identification and analysis of additional DNA markers. The results suggested that insensitivity in the North American isolates was determined by the same locus previously identified in the Dutch and the Mexican strains, which were named *MEX1*, although allelic differences were noted. The insensitivity determinant in a British strain, denoted *MEX2*, proved to be linked to but genetically discrete from *MEX1*.

MATERIALS AND METHODS

Source and manipulation of strains. Isolates of *P. infestans* used in this study are described in Table 1. These were obtained by the authors from infected fields in California or from M. Coffey (University of California, Riverside), Z. Punja (Simon Fraser Uni-

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versity, Burnaby, British Columbia, Canada), R. Shattock (University of Wales, Bangor, United Kingdom), or P. Tooley (USDA-ARS, Fort Detrick, MD). Strains were maintained on rye A agar (2) at 18°C. Mating types were determined by pairing isolates with known A1 or A2 strains. DNA fingerprints were determined as described using RG57 (11,15), which was provided by W. Fry (Cornell University, Ithaca, NY). Sensitivities to metalaxyl were measured as described (9) by growing strains, in duplicate, on rye agar plates containing 0, 0.5, and 5.0 µg (active ingredient) of metalaxyl per ml. Quadruplicate measurements of radial growth were made after 3, 6, 9, and 12 days. Data reported in this paper was based on the 9-day measurements.

Crosses were performed using standard techniques (20). Some crosses were described in an earlier study (9). These included 1114 × 510 (*MEX/mex* × *mex/mex*), which yielded 16 and 11 progeny classified as sensitive and insensitive to metalaxyl, respectively; 88-1-1 × E13a (*MEX/MEX* × *mex/mex*), which yielded 35 F₁ heterozygotes of intermediate insensitivity (*MEX/mex*); and (88-1-1 × E13a) F₁ × Ca65 (*mex/mex*), which resulted in 18 sensitive and 19 insensitive progeny.

DNA markers linked to *MEX*. Loci *W4*, *AI18*, and *AL14* were identified previously (9). Additional loci were identified by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (42) analysis using the bulked segregant analysis technique (29) and 10 nucleotide (nt) primers purchased from Operon Technologies, Inc. (Alameda, CA). Template DNA pools (bulks) were each assembled from six to seven progeny. Reaction products were separated by electrophoresis in Tris-borate-EDTA (TBE; 89 mM Tris, 89 mM H₃BO₃, and 2 mM Na₂EDTA) 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. Analyses of linkage were assisted by the MapManager program (26) and logarithm of the likelihood ratio scores (LOD), Z(θ), were calculated as described (33). Chi-square values were calculated using the Yates correction term. Locus names are based on the RAPD primer used to amplify the band defining the locus.

Mapping assays derived from RAPD markers. For restriction fragment length polymorphism (RFLP) analysis, RAPD bands were first cloned into pCRII (Invitrogen Corp., San Diego, CA). The inserts were subsequently excised, ³²P-labeled, and hybridized to DNA blots as described (19). Mapping was also performed by allele-specific PCR (AS-PCR) or single-strand conformational polymorphism (SSCP) analysis using primers developed by sequencing the cloned RAPD bands and then identifying primer binding sites suitable for amplifying bands from desired genotypes. For SSCP analysis of *AI18*, primers AN18U (5'-TCCTGCGTCTG-GATGATGAT) and AN18L1 (5'-AGCCACTTGTTGAGCAGTGT) were used to amplify bands from both parents. These were resolved by electrophoresis at 5 V/cm at 4°C on a 20-cm 5% poly-

acrylamide gel containing 1/2× TBE and 10% glycerol and visualized by internal labeling with ³²P-dCTP (1 µCi per 25-µl reaction) followed by autoradiography or by staining using SyberGold (Molecular Probes, Eugene, OR). For AS-PCR of *W4*, primers W4U (5'-CGAAATCGTCCTTGAGAA) and W4L (5'-TTCCACGCTG-AGTAACAG) were used to amplify bands from parental DNA. The sequences of parental amplicons were compared to develop allele-specific primer W4U2 (5'-GCAAGCAGGAAGGGAA). W4U2 and W4L were then used to amplify bands from progeny DNA that were resolved using the conditions employed for RAPD analysis.

RESULTS

Metalaxyl-insensitive isolates used in this study. Two insensitive isolates from Canada (115.11 and 135.13) and one from the United States (93H3) were examined, along with three insensitive British, Dutch, and Mexican strains investigated previously (9). Studies of the growth of the North American isolates on rye agar medium containing 0, 0.5, or 5 µg of metalaxyl per ml indicated that each was highly insensitive, because <15% growth inhibition was observed at 5 µg of fungicide per ml (Table 1). This level of insensitivity resembled that of *MEX/MEX* homozygotes such as 88-1-1, while exceeding that of *MEX/mex* heterozygotes such as 1114 and 618. DNA fingerprinting using the RG-57 probe (11) and mating-type assays revealed that the six insensitive isolates were genotypically distinct (Table 1). Only isolate 93H3 matched a common clonal lineage, which was US-7 (11). The DNA fingerprint of 135.1.3 was similar to that of US-8, but these were of opposite mating types (A1 and A2, respectively).

Genetics of insensitivity in North American isolates. The inheritance of insensitivity was studied by mating North American isolates 115.11, 135.13, and 93H3 with various sensitive isolates (Table 1). To determine if oospore-derived colonies were true progeny as opposed to the products of selfing or apomixis, RAPD markers were used to test for the biparental inheritance of genetic material (data not shown).

A cross between isolate 115.11 and sensitive isolate Ca65 yielded 33 authenticated progeny. Our initial hypothesis was that 115.11 would prove to be a *MEX/MEX* homozygote like other highly insensitive strains of *P. infestans* (9,36). However, the segregation data did not fit that model. Instead, 115.11 appeared to be *MEX/mex*, because equal numbers of relatively insensitive and sensitive progeny were recovered from the cross (Fig. 1A). As in previous analyses, phenotypic classes were difficult to distinguish due to the segregation of loci of minor effect (i.e., quantitative trait loci) along with *MEX*. However, prior work suggested that *MEX/mex* and *mex/mex* genotypes were separable at a value of 35% growth at 0.5 µg of metalaxyl per ml relative to unamended media (9). Using this criterion, 18 and 15 insensitive and sensitive progeny, respec-

TABLE 1. Characteristics of isolates of *Phytophthora infestans* referred to in this study

Isolate ^a	Source	Host	Mating type	RG57 genotype ^b	Growth on metalaxyl relative to nonamended media (%) ^c	
					0.5 µg/ml	5 µg/ml
88-1-1	United Kingdom, 1988	<i>Solanum tuberosum</i>	A1	1101110001001101000110011	97 ± 3	83 ± 4
115.11	Alberta, Canada, 1994	<i>Solanum tuberosum</i>	A2	1100110001001101000110011	95 ± 4	87 ± 5
135.13	Alberta, Canada, 1994	<i>Solanum tuberosum</i>	A1	1000100001001101000110111	93 ± 3	94 ± 4
93H3	California, United States, 1993	<i>Lycopersicon esculentum</i>	A2	1000100001001101010110011	92 ± 4	85 ± 7
618	Central Mexico, 1987	<i>Solanum tuberosum</i>	A2	1100110001001101000110011	84 ± 7	29 ± 6
1114	The Netherlands, 1985	<i>Solanum tuberosum</i>	A1	1010111101001101000110011	81 ± 11	52 ± 15
E13a	Egypt, 1984	<i>Solanum tuberosum</i>	A2	1000100001001111000110011	11 ± 9	2 ± 2
1306	California, United States, 1982	<i>Lycopersicon esculentum</i>	A1	1010111001001101000110011	8 ± 3	7 ± 2
510	Central Mexico, 1983	<i>Solanum</i> spp.	A2	1010111001001101010110011	6 ± 2	6 ± 2
Ca65	California, United States, 1981	<i>Lycopersicon esculentum</i>	A1	1010110001001101000110011	4 ± 3	2 ± 2
550	Central Mexico, 1983	<i>Solanum stoloniferum</i>	A2	1011111001001101000110011	4 ± 3	2 ± 2

^a Listed in declining order of relative growth in the presence of 0.5 µg of metalaxyl per ml.

^b As noted by Goodwin et al. (16), band 4 is not reproducible and should not be used for genotype identification.

^c Determined by a radial growth assay. Indicated is the average and standard deviation from at least four replicates.

tively, were calculated that fit the pattern expected for a *MEX/mex* × *mex/mex* cross ($\chi^2 = 0.12$, $P = 0.73$). As indicated below, this was further supported by studying DNA markers linked to *MEX*.

The inheritance of insensitivity in crosses involving isolate 135.13 yielded results similar to those for 115.11. *F*₁ progeny of 135.13 and sensitive isolate 550 displayed phenotypes that ranged between those of the parents (Fig. 1B), suggesting that 135.13 was *MEX/mex*. However, this claim is made tentatively because only six *F*₁ progeny were analyzed because of poor oospore germination and a frequency of selfing or apomixis that greatly exceeded the outcrossing rate. Similar difficulties were observed in crosses between 135.13 and other strains. To enable further genetic studies of the insensitivity determinant from 135.13, an insensitive (135.13 × 550) *F*₁ was crossed to sensitive isolate 510, and 27 progeny were obtained (Fig. 1C). Again using the value of 35% insensitivity to demarcate insensitive and sensitive classes, 14 and 13 offspring were scored. This fit a 1:1 pattern ($\chi^2 = 0.00$, $P = 1.00$) consistent with a *MEX/mex* × *mex/mex* cross. This was later supported using DNA

markers, as indicated below. The continuum of insensitivity levels within the progeny was consistent with the segregation of minor loci, as noted earlier.

Crosses involving isolates of the US-7 lineage such as 93H3 yielded useful but limited results. Oospores from pairings between 93H3 (as well as other US-7 strains) and a variety of sensitive strains germinated poorly, and most oospore-derived cultures appeared to be selfs or apomicts. Nevertheless, five authentic progeny were obtained between 1306 and 93H3. Their levels of insensitivity ($55 \pm 8\%$ growth at 0.5 μ g of metalaxyl per ml compared with unamended media) were intermediate to the parents, which suggested that 93H3 was a *MEX/MEX* homozygote.

Cross between sensitive parents. The strong effect of minor loci in the above crosses raised the issue of whether sensitive parents might yield relatively insensitive progeny. This was tested by crossing two sensitive parents, 1306 and 550, and testing 35 progeny for response to 0.5 μ g of metalaxyl per ml. No offspring showed more than 10% growth at this fungicide concentration compared

Fig. 1. Segregation of metalaxyl insensitivity and DNA markers in *Phytophthora infestans*. The relative levels of insensitivity of parents (open bars) and their progeny (filled bars) are presented as the percent growth on medium amended with 0.5 μ g of metalaxyl per ml relative to that on unamended medium. Error bars represent range of values from four replicates. **A**, Isolates 115.11, Ca65, and their progeny. **B**, Isolates 135.13, 550, and their progeny. **C**, An *F*₁ of 135.13 × Ca65 (indicated by the star in **B**), 510, and their progeny. Indicated below each bar are genotypes determined by random amplified polymorphic DNA analysis of *AN18* or allele-specific polymerase chain reaction mapping of *W4*. Several progeny showed no growth on metalaxyl-amended media and, therefore, no bar is visible. Filled and open circles represent alleles linked to the resistant and sensitive alleles of *MEX*, respectively. Breakpoints between sensitive and insensitive progeny classes, as used for calculations of genetic distance, are indicated by the arrows labeled S and I, respectively.

with unamended media. This suggested that many of the minor loci that segregated in the crosses described earlier may have been epistatic to *MEX* rather than independently contributing to the phenylamide-insensitive phenotype. Similar results were recently reported by Lee and Fry (25).

Isolation of DNA markers linked to *MEX*. Screens for DNA probes linked to insensitivity were executed because such markers could be used to confirm the genetic models described above for 115.11 and 135.13 and determine if the same locus was involved in the North American, British, Dutch, and Mexican isolates. Bulk segregant analysis (29) using the RAPD-PCR method (41) was used for these experiments, employing bulks constructed from individuals of extreme phenotypes as in other studies of quantitative inheritance (41). Two screens were performed, one using the (135.13 $\times$ 550) $F_1 \times$ 510 cross and the other involving a British pedigree, (8811 $\times$ E13a) $F_1 \times$ Ca65. Polymorphisms between the bulks were validated by tests of individual progeny using a $Z(\theta)$ threshold (LOD score) (33) of 3.0.

Two loci, *A11* and *AN18*, were identified by these assays (Figs. 1 to 3). Locus *AN18* was defined by a 1,150-base pair (bp) band that segregated in trans to insensitivity in the (135.13 $\times$ 550) $F_1 \times$ 510 cross (Figs. 1C and 3A). It mapped relative to *MEX* at a recombination fraction (rf) of 0.08, which corresponded to $Z(\theta) = 5.03$. Locus *A11*, represented by a 540-bp band, was linked at rf = 0.08 ($Z(\theta) = 6.62$) in cis to insensitivity in the (8811 $\times$ E13a) $F_1 \times$ Ca65 population (Figs. 2 and 3B).

Comparative genetics of insensitivity. To establish whether or not the same *MEX* locus determined insensitivity in the different isolates, linkages between the DNA and fungicide response loci were measured in the various crosses. These studies integrated the RAPD markers identified above (*A11* and *AN18*) with those reported previously (*AI18*, *AL14*, and *W4*). In most cases, RAPD analysis was uninformative due to nonconservation of the amplification polymorphisms between different crosses. Therefore, mapping assays were also performed using RFLP, AS-PCR, or SSCP methods. The crosses examined addressed insensitivity in North

American (115.11 $\times$ Ca65 and [135.13 $\times$ 550] $F_1 \times$ 510), British ([88-1-1 $\times$ E13a] $F_1 \times$ Ca65), and Dutch (1114 $\times$ 510) isolates. Analysis of the insensitive Mexican isolate, 618, was unnecessary because our previous study indicated that it contained the same insensitivity determinant as isolate 1114.

The results indicated that the major insensitivity determinant in isolates 1114, 618, 115.11, and 135.13 mapped to the same general chromosomal region and likely the same locus, which is hereafter referred to as *MEX1* (Table 2). For example, 115.11 and 135.13 appeared to share the same *MEX* determinant, because *AN18*, which was first detected as a RAPD linked to insensitivity in 135.13, was linked to that trait in 115.11 (linkage in cis at rf = 0.15, $Z(\theta) = 3.83$) (Figs. 1C and 3C). Similarly, marker *W4* was linked to insensitivity in 1114 (rf = 0.10, $Z(\theta) = 6.87$ by RAPD analysis), 618 (rf = 0.12, $Z(\theta) = 3.30$ by RFLP analysis), and 115.11 (rf = 0.09, $Z(\theta) = 5.56$ by AS-PCR) (Fig. 1A). Loci *W4* and *AN18* mapped on opposite sides of *MEX1*.

While *W4* and *AN18* flanked *MEX1* in the isolates described above, this was not the case in British isolate 88-1-1. This suggested that a separate locus was involved, which we denoted *MEX2*. The possibility that a second *MEX* locus existed was proposed in our previous study, in which a statistically significant linkage between insensitivity and *W4* was absent in cross (88-1-1 $\times$ E13a) $F_1 \times$ Ca65. However, a weakness of that study was that data was unavailable for markers on both sides of *MEX1*. This shortcoming could now be rectified using *AN18*, for which linkage to insensitivity in (88-1-1 $\times$ E13a) $F_1 \times$ Ca65 could not be strongly supported ($Z(\theta) = 1.37$) based on SSCP analysis (Figs. 2 and 3F). The linkage measured between *AN18* and *W4* in the British pedigree (rf = 0.19, $Z(\theta) = 3.35$) resembled that assessed in 115.11 (rf = 0.27). Therefore, the absence of an obvious linkage between these markers and insensitivity in 88-1-1 could not be explained by a higher rate of regional recombination or a simple translocation with a breakpoint between *AN18* and *W4*.

MEX1 and *MEX2* appeared linked to each other based upon re-examination of the nature of *W4* and the use of marker *A11*. Addi-

Fig. 2. Analysis of (88-1-1 $\times$ E13a) $F_1 \times$ Ca65 progeny of *Phytophthora infestans*. The two parents (open bars) and their progeny (filled bars) were scored for response to 0.5 μ g of metalaxyl per ml relative to that on unamended medium. Indicated below each bar are genotypes determined by allele-specific polymerase chain reaction analysis of *AN18*, restriction fragment length polymorphism (RFLP) DNA mapping of *W4* (*W4A*), single-strand conformational polymorphism mapping of *W4* (*W4B*), and random amplified polymorphic DNA analysis for *A11*. Breakpoints between sensitive and insensitive progeny classes, as used for calculations of genetic distance, are indicated by the arrows labeled S and I, respectively. Filled and open circles represent alternate alleles of each locus. Linkage between *AL14* and *W4A* was determined by RFLP mapping in an earlier study (9).

Fig. 3. Representative DNA markers used for mapping *MEX* loci in *Phytophthora infestans*. Indicated in the right panels are amplification products from template DNA of representative insensitive (I) and sensitive (S) progeny. The left panels show data for I and S parents (Par.). **A**, Random amplified polymorphic DNA (RAPD) analysis using primer AN18 against (left to right) parents (135.13 × 550) F₁, 510, and their progeny; note that the segregating band is in trans to the insensitive allele. **B**, RAPD analysis using primer A11 against (88-1-1 × E13a) F₁, Ca65, and their progeny. **C**, RAPD mapping of AN18 against 115.11, Ca65, and their progeny. **D**, Restriction fragment length polymorphism (RFLP) mapping *W4* by RFLP analysis using the same strains as in **B**. **E**, Allele-specific polymerase chain reaction mapping of *W4* using the same strains as in **B**. **F**, Single-strand conformational polymorphism (SSCP) mapping of AN18 using the same strains as in **B**. In each panel, the size of the relevant band is indicated in the left margin in kilobases (kb) except for **F**, in which an arrow marks the relevant SSCP allele derived from a 0.22-kb amplicon.

tional mapping studies revealed that two genetically separate *W4* loci existed in *P. infestans*, denoted *W4A* and *W4B* (Figs. 2 and 4). That multiple loci existed was first suspected from RFLP analyses, in which one parent commonly displayed more bands than the other. This is illustrated in Figure 3D, in which the (88-1-1 × E13a) F₁ strain shows more bands than Ca65. A similar excess of bands was seen in 12 of 39 restriction digests tested, while Ca65 never contained more bands than the F₁ strain in any digest. To help determine if multiple loci existed, *W4* was mapped both by

Fig. 4. Map of chromosomal region containing *MEX* loci of *Phytophthora infestans*. Indicated are the linkage relationships (as recombination fraction, rf) between insensitivity loci *MEX1*, *MEX2*, and the DNA markers described in text. All linkages are significant with $Z(\theta) > 3.0$. The map presents a consensus of data from the crosses described in this study and, therefore, does not indicate that *MEX1* and *MEX2* are necessarily present in all isolates.

the RFLP method (by hybridization with the cloned *W4* RAPD band) (Fig. 3D) and the AS-PCR method (using primers based on the sequence of that band) (Fig. 3E). This revealed that two discrete *W4* loci existed that were linked at $rf = 0.16$ ($Z[\theta] = 4.02$). Moreover, the locus mapped by AS-PCR, named *W4B*, was linked to *A11* at $rf = 0.11$ ($Z[\theta] = 5.65$). When data for all of the markers in (88-1-1 × E13a) F₁ × Ca65 were examined, it was apparent that all could be grouped into a statistically robust linkage group for which a consensus map is shown in Figure 4.

The wide duplication of *W4* throughout the species was suspected, since supernumerary RFLP bands were detected in comparisons between other isolates including 510, 618, 115.11, and 1114. However, the presence of the extra band was not generally associated with insensitivity.

DISCUSSION

A diversity of genetic mechanisms contribute to phenylamide insensitivity in *P. infestans*. First, insensitivity is inherited as a quantitative trait in which major *MEX* loci interact with genes of minor effect. Most of the minor genes are likely epistatic to *MEX*, because offspring of sensitive parents showed little variation in metalaxyl response. Second, at least two major genetic loci appear to be present, which we have named *MEX1* and *MEX2*. Third, allelic differences probably exist at *MEX1*, as evidenced by the higher levels of insensitivity exhibited by *MEX1/mex1* heterozygotes from the Canadian isolates versus heterozygous Dutch and Mexican isolates.

These conclusions can be interpreted within the context of observations made since phenylamides first came into common use in the 1970s. In retrospect, that multiple loci influence sensitivity might have been expected, because *P. infestans* isolates obtained before and after the commercial release of phenylamides vary in their response to such compounds (17,28,38). The existence of multiple *MEX* genes, or allelic differences at the same locus, was also suggested by a report stating that different metalaxyl-insensitive isolates of *Peronospora parasitica* were unequally insensitive to another phenylamide, cyprofuram (31). Nevertheless, most previous genetic studies of phenylamide insensitivity invoked simple models involving one locus (1,3,6,27,36). Shaw (37) did suggest that more than one *MEX* locus might exist in *P. infestans* after observing that some selfed strains were less sensitive than their progenitor (37); a *MEX1MEX2/mex1mex2* genotype was proposed for the progenitor to explain this, although our data suggest that segregation of minor loci could also have been responsible.

The genetic complexity of mechanisms conditioning insensitivity complicates studies of the evolution and distribution of the trait within *P. infestans*. In recognition of quantitative variation between isolates, epidemiological studies have started to report insensitivity phenotypes in increasing detail. For example, while some early studies simply distinguished resistant versus sensitive types, more recent publications placed isolates in an additional intermediate category or in as many as 20 grades from full sensitivity to resistance (14,17). Such detailed presentations of phenotypes helps to reveal the diversity of mechanisms by which insensitivity evolves. For example, even within the US-8 clonal lineage, different isolates were reported to show from 5 to 60% reductions in growth at 5 µg of metalaxyl per ml (17). This may imply that additional mutations have accumulated within the lineage.

TABLE 2. Summary of genetic analysis of metalaxyl-insensitive isolates of *Phytophthora infestans*

Isolate	Genotype	Genetic distance between DNA marker and major insensitivity determinant ^a				
		<i>AI18</i>	<i>AN18</i>	<i>W4</i>	<i>AL14</i>	<i>AI1</i>
1114	<i>MEX1/mex1</i>	0.14 (RAPD)	...	0.11 (RAPD)	0.18 (RAPD)	...
115.11	<i>MEX1/mex1</i>	0.18 (RAPD)	0.15 (RAPD)	0.09 (RFLP)	...	...
135.13	<i>MEX1/mex1</i>	... ^b	0.08 (RAPD)	...	...	...
618	<i>MEX1/mex1</i>	...	...	0.12 (RFLP)	...	...
88-1-1	<i>MEX2/mex2</i>	...	0.54 (SSCP) ^c	0.35 (RFLP) ^d	0.35 (RFLP)	0.08 (RAPD)
				0.19 (AS-PCR) ^d		

^a Genetic distances are presented as recombination fraction. The type of assay used for mapping is indicated in parentheses. RAPD = random amplified polymorphic DNA; RFLP = restriction fragment length polymorphism; SSCP = single-strand conformational polymorphism; and AS-PCR = allele-specific polymerase chain reaction.

^b Not determined.

^c Sum of distances from *MEX2*.

^d Two loci were identified using probes based on the *W4* RAPD band. The loci defined by RFLP and AS-PCR analyses are named *W4A* and *W4B*, respectively.

Insensitivity likely evolves stepwise starting from mutation at a *MEX* locus, with the genetic background influencing the quantitative level of the trait. The insensitivity of such a *MEX/mex* strain could rise (or fall) if additional changes occurred, with selection mediated by fungicide use. For example, a *MEX/MEX* strain such as 88-1-1 could develop from a heterozygote through mutation, gene conversion, selfing, or mating with an insensitive partner. Our analysis suggests that homozygosity is not a prerequisite for high insensitivity, however, and events at minor loci could also lead to elevated insensitivity levels. Further elaboration of the mechanism by which insensitivity evolves is of interest not only in regards to phenylamides but also for predicting the future responses of *P. infestans* to other fungicides.

That *MEX1* and *MEX2* are linked is consistent with observations from other species in which genes of related function are often close to each other. For example, pathogen resistance genes in plants (5), homeobox genes in insects (21), and drug resistance genes in humans (24) frequently occur in clusters. This usually reflects the chromosomal mechanisms such as unequal crossing over or duplication by which such families evolved. Genes that are structurally unrelated but function in the same metabolic or developmental pathway can also be linked, as in the case of nitrate-assimilation (22) and sporulation-related genes in some fungi (18). This may reflect a cellular advantage of coordinately regulating genes in the same chromatin domain or nuclear compartment (13).

Our experiments benefited greatly from having DNA markers linked to *MEX*. Such probes helped define the basis of insensitivity, particularly in crosses such as 115.11 × Ca65, in which support for a major locus based on phenotype alone would have been tenuous due to the continuum of insensitivity levels displayed by progeny. The DNA markers were also useful in indicating that *MEX1* and *MEX2* were separate, but linked. Limitations of the analysis must be recognized, however. For example, the assignment of *MEX1* to the *AN18-W4* interval does not exclude the possibility that the locus comprises several tightly linked genes. Also, while the chromosomal locations of *MEX1* and *MEX2* were distinct, it does not follow that they must encode proteins of different function. For example, mutations in either of several tubulin genes present in some fungi confer resistance to benzimidazoles (23). Also, our genetic analysis does not exclude the possibility that *MEX1* and *MEX2* represent the same gene, which transposed between sites in different isolates. The *MEX* genes themselves will be required to address such issues, and the isolation of the genes by positional cloning is now underway in our laboratory.

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