Techniques

The Use of Allele-Specific Oligonucleotide Probes to Characterize Resistance to Benomyl in Field Strains of *Venturia inaequalis*

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ABSTRACT

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A procedure was developed for detecting point mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis*, using the polymerase chain reaction (PCR) in combination with allele-specific oligonucleotide (ASO) analysis. PCR was used to amplify a specific 1,191-bp DNA sequence of the beta-tubulin gene in DNA extracts from axenically grown mycelium or individual apple scab lesions. The amplified DNA sequence was then probed with 18-mer end-labeled oligonucleotides specific for the sensitive phenotype or for three benomyl-resistant phenotypes in strains of *V. inaequalis*. The point mutations, converting codon 198 from glutamic acid in the sensitive strain to lysine or alanine, were detected by ASO analysis in highly resistant and very highly resistant strains, respectively. A point mutation, converting codon 200 for phenylalanine in sensitive strains to tyrosine, was detected by ASO analysis in all medium resistant strains but one from Chile, isolate CHILE 24B. Sequence analysis revealed that an alternate codon for tyrosine was present in the Chilean isolate. Therefore, the mutation in isolate CHILE 24B was not detected by any of the four ASO probes used in this study. ASO analysis was a useful tool for detecting and characterizing benomyl-resistant strains of *V. inaequalis* and could be expanded to other plant pathogenic fungi.

Benzimidazole compounds are widely used as fungicides and anthelmintics and are also promising antineoplastic agents (3). Inherent in the site-specific mode of action of benzimidazole compounds is the potential for developing resistance in target organisms. Point mutations in the beta-tubulin gene have been shown to confer resistance to the benzimidazole fungicide benomyl (5,8,11,13,20).

Benomyl-resistant strains of *Venturia inaequalis* (Cooke) G. Wint. have been isolated in many apple growing regions worldwide. In addition to sensitive (S) strains, four classes of resistant strains (low [LR], medium [MR], high [HR], and very highly resistant [VHR]) were established, based on the growth response of the strains on media amended with benomyl (9,14,17). Moreover, HR and VHR strains were differentiated from each other

according to sensitivity of VHR strains to diethofencarb (7,15). Studies on the genetic basis of benomyl resistance indicated that this resistance in *Venturia* was determined by a single gene (9,14,16,17). Sequence analysis of the beta-tubulin DNA of field strains of *V. inaequalis* revealed that point mutations in codons 198 or 200 were associated with differential resistance to benomyl. Conversion of codon 200 from phenylalanine in a sensitive strain to tyrosine was associated with medium resistance to benomyl. Conversion of codon 198 from glutamic acid to lysine or alanine was associated with high or very high resistance to benomyl, respectively (11).

DNA probes have been developed to detect bacteria with resistance to copper or streptomycin (2,6,12). Unlike detection of benomyl resistance, in which point mutations are responsible for resistance, the detection of copper or streptomycin resistance is based on the presence of genes that are absent in sensitive strains. Genetic disorders caused by point mutations in DNA of humans are now detected by the combined use of the polymerase chain reaction (PCR)(4) and allele-specific oligonucleotide (ASO) analysis (1,19). PCR is used to amplify the appropriate DNA sequence, and then ASO analysis is used to detect the single base pair mutations.

The objective of this study was to develop ASO analysis for the rapid detection of point mutations that confer resistance to benomyl in field strains of *V. inaequalis*. ASO analysis was used to establish the relatedness of the allelic mutations in a collection of field strains isolated in different regions of the world. Individual apple scab lesions were subjected to ASO analysis to characterize allelic mutations in strains from an orchard in which benomyl had not been applied for more than 10 yr.

MATERIALS AND METHODS

Fungal strains. The single-spore isolates of *V. inaequalis* used in this study were from a large collection of field strains previously characterized in studies on the inheritance of benomyl resistance and negatively correlated cross-resistance to diethofencarb (7,17). The strains originated in different geographic regions of the world and exhibited widely varying levels of resistance to benomyl. Strains IL-2, IL-3, IL-6, IL-7, and IL-8 of *V. pirina* Aderhold were provided by E. Shabi, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. Each strain was designated as sensitive (S), low resistance (LR), medium resistance (MR), high resistance (HR), or very high resistance (VHR) to benomyl according to its growth response on media amended with benomyl or diethofencarb (6,9,14–16).

A. Allele-specific oligonucleotide probes for *Venturia inaequalis*

				198		200		
ASO^{S-LR}	5' C	TCT	GAC	GAG	ACA	TTC	TG 3'	
ASO^{MR}				C	GAG	ACA	TAC	TGC ATT GA
ASO^{HR}		C	TCT	GAC	AAG	ACA	TTC	TG
ASO^{VHR}		C	TCT	GAC	GCG	ACA	TTC	TG

B. Sequence for codons 195 to 203 of the beta-tubulin gene

									Number of mismatches
Venturia inaequalis	AAC	TCT	GAC	GAG	ACA	TTC	TGC	ATT	GAC	0
V. pirina	AAT	TCG	GAC	GAG	ACC	TTC	TGC	ATT	GAC	3
Monilinia fructicola	AAC	TCT	GAT	GAA	ACC	TTC	TGT	ATC	GAT	3
Penicillium aurantiogriseum	CAC	TCC	GAC	GAG	ACC	TTC	TGT	ATC	GAT	2
P. digitatum	CAC	TCC	GAC	GAG	ACT	TTC	TGT	ATC	GAT	2
P. expansum	CAC	TCC	GAC	GAG	ACT	TTC	TGT	ATC	GAT	2
P. italicum	CAC	TCC	GAC	GAG	ACT	TTC	TGT	ATC	GAT	2
P. puberulum	CAC	TCC	GAC	GAG	ACC	TTC	TGT	ATC	GAT	2
P. viridicatum	CAC	TCC	GAC	GAG	ACC	TTC	TGT	ATC	GAT	2
Sclerotinia homoeocarpa	AAC	TCT	GAC	GAG	ACC	TTC	TGT	ATC	GAT	1

Fig. 1. A, Sequence of allele-specific oligonucleotide (ASO) probes for *Venturia inaequalis*. The ASO probes for medium (MR), high (HR), and very high resistance (VHR) to benomyl differ from the beta-tubulin DNA for sensitive (S) and low-resistance (LR) strains by one nucleotide (arrows). **B,** Sequence of codons 195–203 of the beta-tubulin gene from benomyl-sensitive field strains of 10 plant pathogenic fungi. Under conditions of high stringency, the ASO^{S-LR} probe will be removed from its complementary antisense strand sequence from fungi other than *V. inaequalis* because of one to three mismatches (underlined) in the third position of codons.

DNA isolation and polymerase chain reaction. Total DNA was isolated from mycelium from cultures grown in potato-dextrose broth or on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) as previously described (11), except that the mycelium was macerated with disposable pellet pestles (Kontes, Morton Grove, IL) in microcentrifuge tubes. The isolated DNA was then used as template in the PCR. Two 22-mer oligonucleotides, 5'-CAAACCATCTCTGGCGAACACACG and 5'-TGGAGGA-CATCTTAAGACCACG, which were identical in sequence to codons 22–28 and complementary in sequence to codons 359–365 of the beta-tubulin gene of *V. inaequalis*, respectively, were used as PCR primers to amplify a 1,191-bp DNA sequence. PCR was performed, and amplification products were analyzed by agarose gel electrophoresis as described previously (11).

ASO probes. Four ASO probes for detecting allelic mutations in the beta-tubulin gene of *V. inaequalis* were synthesized in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University, East Lansing. The 18-mer oligonucleotides were designated as ASO^{S-LR}, ASO^{MR}, ASO^{HR}, and ASO^{VHR} probes according to the specificity of each probe. Each ASO probe included the sequences of codons 198 and 200 (Fig. 1A) because mutations in these codons were associated with resistance to benomyl in field strains of *V. inaequalis* (11). ASO probes were end-labeled with gamma-^{32}P-ATP (Du Pont, Boston, MA), using T4 polynucleotide kinase (Promega Corporation, Madison, WI) to a minimum specific activity of 2×10^9 dpm/pmol.

ASO analysis. PCR-amplified beta-tubulin DNA (25 ng per sample) was denatured in 0.25 N NaOH for 10 min and then applied to a nylon membrane (GeneScreen-Plus, Du Pont, Boston, MA) in a dot blot manifold. The dot blots were incubated in prehybridization solution (1 M NaCl, 50 mM Tris-HCl, pH 7.5, 10% dextran sulfate, 1% SDS, 0.2% Ficoll (MW 400,000), 0.2% polyvinylpyrrolidone (MW 40,000), 0.2% bovine serum albumin, 0.1% sodium pyrophosphate, and 0.25 mg/ml denatured salmon sperm DNA) for 2 h at 65 C according to the manufacturer's procedure. An end-labeled ASO probe was then added to the prehybridization solution and incubated at 37 C for at least 4 h. The blots were washed three times for 15 min each in 2× SSC buffer (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 6.8) at room temperature. A high-stringency wash, three times for 2 min each in 2× SSC buffer, was then used to remove ASO probe with a single base pair mismatch from the blots. The optimum temperature for the high-stringency wash with each probe was determined empirically. The dot blots were exposed to X-ray film for 0.5–2 days at −70 C.

Direct characterization of alleles in apple scab lesions. Leaves and fruits with scab lesions were collected in an apple orchard at the Botany and Plant Pathology Farm on the campus of Michigan State University, East Lansing. Monitoring of the apple scab population in 1976 revealed the existence of an S and VHR population in this orchard (Jones, *unpublished*). The resistance level of spores from individual lesions was determined by plating conidia from each lesion on PDA amended with 0, 0.5, 5, or 50 mg/L of benomyl or 1 mg/L of diethofencarb (7,11,17). Lesions were cut from leaves or fruits with a scalpel and individually transferred to microcentrifuge tubes. Isolation of DNA from the lesions, amplification of the target sequence by the PCR, and ASO analysis were done as described above. DNA isolated from healthy leaves and fruits was included as a control to determine whether there was significant PCR amplification of other DNA sequences.

RESULTS

ASO analysis. DNA isolated from as little as 10 mg fresh weight of mycelium from broth or agar plate cultures was sufficient for

the amplification of the 1,191-bp target sequence of the beta-tubulin gene using PCR. Amplified DNA was not detected when sample DNA was omitted from the PCR reaction mixture.

Each of the four ASO probes hybridized to the PCR-amplified beta-tubulin DNA of all strains of *V. inaequalis* after washing the dot blots at 50 C (Fig. 2A). After washing at 64 C, the ASO^{S-LR} probe hybridized only with the PCR amplified beta-tubulin DNA of strains with S or LR phenotype and not to DNA of strains with the MR, HR, or VHR phenotype (Fig. 2B and Table 1). The ASO^{MR}, ASO^{HR}, and ASO^{VHR} probes exhibited allele-specific hybridization after washing at 61, 61, and 63 C, respectively (Fig. 2C–E and Table 1).

Amplified beta-tubulin DNA from strain CHILE 24B of *V. inaequalis* with medium resistance to benomyl did not hybridize with the ASO^{MR} probe, nor with the ASO^{S-LR}, ASO^{HR}, or ASO^{VHR} probes under conditions of high stringency. Amplified beta-tubulin DNA from strain CHILE 24B was sequenced and contained a mutation in codon 200 of the beta-tubulin gene. The codon for phenylalanine (TTC) found in sensitive strains was replaced by a codon for tyrosine (TAT) in strain CHILE 24B.

Also, none of the four ASO probes hybridized with amplified beta-tubulin DNA from strains of *V. pirina* with the S, MR, HR, or VHR phenotype. When the sequence of the ASO^{S-LR} probe was compared to the corresponding sequence from a sensitive strain of *V. pirina*, there were different nucleotides in the third position of three codons. A comparison of the sequence of ASO^{S-LR} with sequence data for a sensitive strain of *Sclerotinia homoeocarpa, Monilinia fructicola*, and six species of *Penicillium* showed a different nucleotide in the third position of one, three, and two codons, respectively (Fig. 1B).

Direct characterization of alleles in apple scab lesions. Electrophoresis showed that a 1,191-bp DNA fragment was amplified in PCR reactions with DNA extracts from 12 apple scab lesions, but no amplification of DNA was detected in PCR reactions with DNA extracts from five healthy leaves and from five healthy fruits. The ASO^{S-LR} probe hybridized with amplified DNA from seven lesions and the ASO^{VHR} probe hybridized with amplified DNA from five lesions. The ASO^{MR} and ASO^{HR} probes did not hybridize with amplified DNA from any of the lesions.

Conidia from all 12 scab lesions germinated and formed colonies on PDA. Conidia from seven lesions were characterized as sensitive to benomyl because they failed to grow on PDA amended with 0.5 mg/L of benomyl. The benomyl phenotype of the scab fungus in the remaining five lesions was VHR because conidia from each lesion grew on PDA amended with 50 mg/L benomyl

TABLE 1. Benomyl resistance phenotypes of field strains of *Venturia inaequalis* from different geographic regions and hybridization of a series of allele-specific oligonucleotide (ASO) probes to beta-tubulin DNA from each strain

Strain designation, origin	Resistance to benomyl[a]	ASO probe			
		S-LR	MR	HR	VHR
B7, Germany	S	+[b]	−	−	−
MSU-18, Michigan	S	+	−	−	−
WC, Michigan	S	+	−	−	−
W8, Germany	S	+	−	−	−
W12, Germany	S	+	−	−	−
#42 MINNS 118, New York	LR	+	−	−	−
#6 MITCHELL 94-1, New York	LR	+	−	−	−
MSU-20, Michigan	LR	+	−	−	−
CHILE 24B, Chile	MR	−	−[c]	−	−
MAINE 4, Maine	MR	−	+	−	−
MAINE 8, Maine	MR	−	+	−	−
V-560, Maine	MR	−	+	−	−
SIS-16, Maine	MR	−	+	−	−
RI, Australia	HR	−	−	+	−
I-23, Israel	HR	−	−	+	−
I4-1, Italy	HR	−	−	+	−
75-71, New Zealand	HR	−	−	+	−
RH-4, Michigan	HR	−	−	+	−
TUFRI, France	VHR	−	−	−	+
TU86R2, France	VHR	−	−	−	+
I-22, Israel	VHR	−	−	−	+
KV3C, Michigan	VHR	−	−	−	+
MSU-33, Michigan	VHR	−	−	−	+
75-26, New Zealand	VHR	−	−	−	+
SH-2, Michigan	VHR	−	−	−	+

[a] S = sensitive to benomyl at 0.5 mg/L; LR = low resistance, growth at 0.5 mg/L but not at 5 mg/L; MR = medium resistance, growth at 5 mg/L but not at 50 mg/L; HR = high resistance, growth at 50 mg/L and insensitive to diethofencarb at 1 mg/L; VHR = very high resistance, growth at 50 mg/L but sensitive to diethofencarb at 1 mg/L.

[b] + = Hybridization, − = no hybridization with ASO probe.

[c] CHILE 24B had an alternate codon for tyrosine (see text).

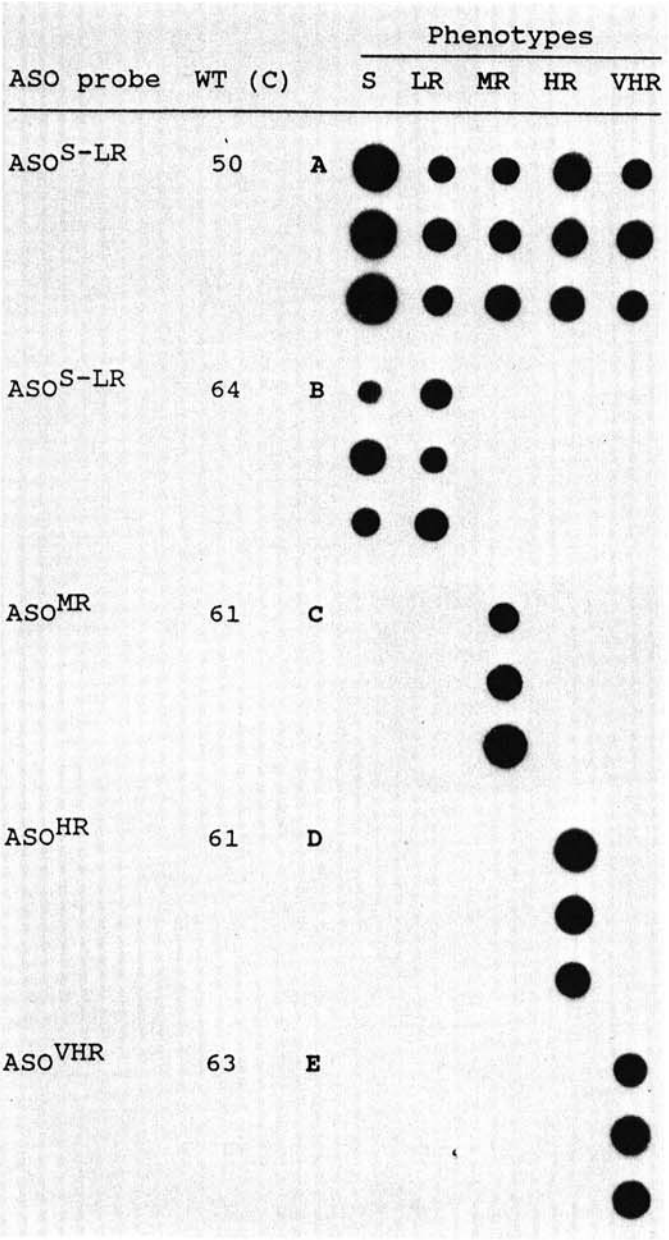

Fig. 2. Allele-specific oligonucleotide analysis with amplified beta-tubulin DNA of *Venturia inaequalis*. The ASO^{S-LR} probe hybridized to DNA on dot blots in panels A and B, and the blots were subsequently washed at a temperature (WT) of low stringency (A) or optimum stringency (B). The ASO^{MR}, ASO^{HR}, and ASO^{VHR} probes were hybridized to DNA on the blots in panels C, D, and E, respectively. The blots were subsequently washed at optimum stringency. Amplified beta-tubulin DNA from sensitive, low, medium, high, and very high resistant strains was applied to each blot in column S, LR, MR, HR, and VHR, respectively. S strains (from top to bottom): B7, MSU-18, and WC; LR strains: MINNS 118, MITCHELL 94-1, and MSU-20. MR strains; MAINE 4, MAINE 8, and SIS-16; HR strains: RI, I-23, and RH-4; and VHR strains: TU86R2, 75-26, and KV3C.

but not on PDA amended with 1 mg/L diethofencarb. The phenotypes determined by ASO analysis were in agreement with the phenotypes determined by growing the fungus from each lesion on benomyl and diethofencarb-amended media.

DISCUSSION

The results of ASO analysis performed on amplified beta-tubulin DNA from several benomyl-resistant field strains of *V. inaequalis* that had not been previously examined were in agreement with those based on sequence analysis of beta-tubulin DNA from single strains of each resistance phenotype (11). Codons for lysine and alanine at position 198 were always associated with the HR and VHR phenotypes, respectively, and base substitutions giving a tyrosine codon at position 200 were always associated with the MR phenotype. The independent selection of identical codon conversions in beta-tubulin DNA of field strains from diverse geographic regions provides additional evidence that these mutations are the primary basis for resistance to benomyl. None of the ASO probes hybridized with amplified DNA from MR strain CHILE 24B of *V. inaequalis*, indicating that a previously uncharacterized mutation was present in the 18-bp sequence of this beta-tubulin gene. It was subsequently determined that an alternate codon for tyrosine, TAT rather than TAC, was present in the beta-tubulin gene of CHILE 24B. In a previous study (11), we determined the single base pair mutations that were associated with benomyl resistance in strains of *V. inaequalis* and *V. pirina* with MR, HR, and VHR phenotypes. However, no mutation was detected in strains with the LR phenotype. Until the molecular basis for the LR phenotype is established, it will be impossible to distinguish between S and LR strains by ASO analysis.

ASO analysis involves the following steps: extracting DNA, amplifying the target sequence using PCR, and probing amplified DNA with allele-specific probes. A rapid and simple mini-preparation extraction procedure was developed to reduce the chance of contaminating the extraction mixture with DNA that could serve as template during PCR amplification. Amplification of beta-tubulin DNA by PCR alleviated the need to obtain large amounts of mycelium for DNA extraction. After hybridizing the probe with amplified DNA at 37 C, dot blots were washed at an elevated temperature to remove probes with a single base pair mismatch. The optimum wash temperature for removing nonspecific probes varied for each ASO probe and was related to the guanine plus cytosine content of the probes.

With ASO analysis, the sequence must be known before PCR primers and ASO probes can be designed. A similar restriction holds for the recently developed technique of allele-specific amplification by PCR, in which an even quicker characterization of single base pair mutations is possible without the use of radiolabelled probes (18,21). Also, degeneracy in the genetic code interfered with the characterization of allelic mutations in the beta-tubulin DNA of other plant pathogenic fungi. For example, none of the *V. inaequalis*-specific ASO probes hybridized with amplified beta-tubulin DNA from *V. pirina*, because of variations in the third position of three codons (Fig. 1B). A set of species-specific probes could circumvent this limitation of ASO analysis.

ASO analysis is a rapid method for characterizing point mutations in beta-tubulin alleles that confer resistance to benomyl in field strains of *V. inaequalis*. The method is sensitive and versatile. Both axenic mycelium and tissue from apple scab lesions can be assayed. Because ASO analysis can be performed on material from individual lesions, as demonstrated in this study, this method could be employed to identify and characterize benomyl resistance in pathogens that are difficult to culture and in obligate parasites, such as powdery mildews.

Provided that resistance is based on point mutations, ASO analysis might also assist in determining the potential variability of target sites for new pesticides with a site-specific mode of action. The key to a successful analysis of the potential variability of an unknown target site is the rapid cloning of the gene that confers resistance. Efficient gene transfer systems for saprophytic and plant pathogenic fungi are now available and can be used to isolate resistance genes as demonstrated with *Ustilago maydis* (10). Characterization of point mutations in a large collection of laboratory-induced mutants is likely to indicate the potential variability of a target site, as in the case of benomyl (11), and will be facilitated by ASO analysis. A comparison of the codon conversions in the beta-tubulin gene of field strains with laboratory-induced mutants of saprophytic fungi showed that mutations conferring field resistance represent a subset of the mutations recovered in laboratory experiments. It was hypothesized that mutations, which were recovered only in a laboratory environment, might interfere with fitness of the mutant strains (11). To investigate fitness, it will be necessary to determine which of the laboratory-induced mutants could survive under field conditions, and which mutations could only survive in the laboratory. ASO analysis might be used to monitor the survival of laboratory-induced mutants on plants in growth chamber experiments involving mixed populations of a pathogen.

In conclusion, we found that ASO analysis was an easy, rapid method and believe that it can be readily adapted to a range of tests to determine relevant single base pair mutations in plant pathogenic organisms. With this method, we have demonstrated that, with one exception, strains of *V. inaequalis* from diverse locations but with similar levels of benomyl resistance carry the same mutations in the beta-tubulin gene.

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