Using variable number of tandem repeats (VNTRs) to develop a species-specific DNA diagnostic

Eric W.A. Boehm1, Zhonghua Ma2 & Themis J. Michailides2

1Dept. of Biology, Kean University, Union, NJ; 2Plant Pathology, University of California Davis.

Background: Brown rot of stone fruits (Prunus sp.), caused by the ascomycete fungus Monilinia fructicola (Wint.) Honey, is a serious disease in the California central valley & often results in severe post-harvest yield losses, in some cases in excess of 30%. Currently, an estimated 100,000 ha are under cultivation in the Sacramento and San Joaquin Valleys, producing annually some 800,000 t of stone fruit (peaches, plums, nectarines, apricots & prunes), valued at over 700 million dollars. Currently, California growers expend an enormous amount of capital to spray fungicides as a prophylactic measure whenever weather conditions are deemed favorable for disease development. A need therefore exists to be able to predict in advance whether a particular orchard carries a disease load and is therefore a good candidate for spraying or whether the orchard carries a minimal load & can therefore avoid the need for spraying. At the moment there is no way reliable way to do this.

The development of a species-specific molecular diagnostic capable of detecting M. fructicola from infected host tissues could help the California Stone Fruit Industry to (1) predict the incidence of disease prior to the onset of visual symptoms; (2) determine the efficacy of various chemical & cultural control strategies; (3) evaluate the efficacy of post-harvest storage techniques; & (4) establish appropriate quarantine measures based on a species-specific diagnostic. Currently, M. fructicola is a quarantine listed organism in Europe. The diagnostic ideally would be used to sample orchard flowers in the early spring, prior to onset of fruit, to determine which orchard requires fungicide spraying. The present study2 was initiated to develop a series of DNA diagnostics, based on both full-length hybridization probes and derived PCR oligonucleotide primer sets, capable of detecting the stone fruit pathogen Monilinia fructicola to the exclusion of all the other associated microflora on the flower and fruit surface.

The cloning strategy: Variable number of tandem repeats (VNTRs) are highly repetitive, tandem DNA sequences that are found scattered throughout the genome of most eukaryotes analyzed. They are non-coding and therefore under no selective pressure to maintain sequence conservancy. Thus, they are ideal candidates for molecular cloning because these very highly repeated DNA fragments have been shown to be a useful diagnostic at the species level3. Their function is unknown. The hybridization strategy used to identify species-specific repetitive clones in this study was threefold: (1) identification of highly repeated plasmid clones in a genomic library using total DNA as the probe source on colony lift membranes; (2) exclusion of ribosomal clones using the entire 9kb ribosomal repeat from a related fungus as a heterologous probe; and (3) determination of specificity and sensitivity of the identified repeat clones. Under nonsaturating probe concentrations & high stringency hybridization and wash temperatures, plasmid clones containing highly repeated fungal sequences generated stronger signals than clones containing moderately to low- or single-copy fungal sequences when probed with total genomic DNA. This difference was the basis for the initial screen.

Fig. 1. (A) Cloning strategy: 12 genomic plasmid clones with released insert(s) of fungal DNA. B. The same hybridized with the heterologous ribosomal clone. C. The same hybridized with total DNA as the probe source. Clones 73, 150 & 210 are non-ribosomal & carry fungal VNTRs. They are possible candidates for the development of a species specific diagnostic.

Fig. 2. (right). Dot blot hybridization species specificity survey. 96 fungal isolates (8 fungal species) commonly associated with stone fruit were challenged with the cloned VNTR probes pMF73 & pMF210. The probes only recognized the target fungal species, Monilinia fructicola (in red), to the exclusion of all other fungal species commonly associated with stone fruit. In addition to CA isolates, the specificity extended to include isolates from MI, OR, GA & AUST. Thus, these two probes seem to be species specific to a broad geographic range of fungal isolates of M. fructicola.

Fig. 3. (left). PCR species specificity survey. Forward and reverse species-specific oligonucleotide primers were designed to assess the limits of specificity for a subset of the same isolates screened above by dot blot hybridization. Using high annealing temperatures, the same specificity profiles were again observed for the same isolates. The specificity was restricted to M. fructicola from the same broad geographic range.

Fig. 4. (far left below). Quantitative dot blot sensitivity assay and reconstruction experiment. A concentration series, in pg of DNA, was prepared from both the gel purified pMF73 insert and fungal genomic DNA & hybridized to the insert. Copy number estimate was derived by comparing the signal intensities of fungal DNA to that of the insert using quantitative image analysis software. The signal intensity obtained with 50pg of genomic DNA corresponded to that with 1pg of the 204bp insert. The sequence was calculated to comprise approximately 2% of the genome. Dot blot sensitivity thresholds indicated that the VNTR pMF73 was capable of detecting as little as 50pg of fungal genomic DNA.

Fig. 5. (near left). PCR sensitivity thresholds. To determine the detection limits of the species-specific primer pairs, sensitivity assays were done for known concentrations of fungal DNA template and template derived from known concentrations of fungal DNA template. Species-specific primer pair sensitivity, at high annealing temperatures, was capable of detecting 50pg of fungal DNA, which corresponded to a sensitivity threshold of approximately 10 fungal spores. Note the dramatic difference in sensitivity levels between dot blot hybridization (Fig. 4) versus that obtained by PCR (Fig. 5).

Fig. 6. (right) In planta dot blot detection. Dot blot detection of Monilinia fructicola from isolated stone fruit (Prunus) flowers. Flowers with obvious symptoms indicated by (+), without symptoms (+/-) and very young flowers (-). The species-specific DNA diagnostic is capable of detecting the fungus in flowers prior to onset of visible symptoms. The molecular diagnostic therefore provides a prediction of orchard disease load prior to the onset of fruit, thus allowing ample opportunity to field evaluate orchards before fungicide spraying.

Literature Cited: