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Identification of DNA Unique to the Tomato Fusarium Wilt and Crown Rot Pathogens

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Abstract.

Wilt and crown rot are two important diseases of tomato caused by different strains ("*formae speciales*") of the fungus, *Fusarium oxysporum*. While both pathogens are members of the same fungal species, each differs genetically and resistance to the diseases is controlled by different genes in the plant. Additionally, the *formae speciales* differ in their ecology (e.g. optimal temperature of disease development) and epidemiology. Nevertheless, the distinction between these diseases based on symptoms alone may be unclear due to overlapping symptomatology. We have found in our research that the ambiguity of the pathogens is further confounded because strains causing tomato wilt or crown rot each may belong to several genetically and phylogenetically distinct lineages of *F. oxysporum*. Furthermore, individual lineages of the pathogen causing wilt or crown rot may themselves be very closely related. The diseases share the characteristic that the pathogen's inoculum may be aurally dispersed. This work has revealed a complex evolutionary relationship among lineages of the pathogens that makes development of molecular diagnostic methods more difficult than originally anticipated. However the degree of diversity found in these soil-borne pathogens has allowed study of their population genetics and patterns of dispersal in agricultural settings.

Objectives of original proposal

The objectives of the original proposal (revised in part in accordance with the BARD review panel) were to 1) isolate DNA sequences that are specific to *F. oxysporum* f. sp. *lycopersici* (the tomato wilt pathogen), *F. oxysporum* f. sp. *radicis-lycopersici* (the tomato crown rot pathogen) or vegetative compatibility groups within these *formae speciales* of *F. oxysporum*, 2) localize these pathogen-associated sequences to particular fungal chromosomes by Southern hybridization to chromosome-sized DNAs of these fungi separated by pulsed-field electrophoresis, and 3) sequence pathogen-associated clones to determine their content and origin. Publications aimed at accomplishing those goals are listed and discussed below.

Relevant data, methodology, results and discussion.

In order to construct molecular diagnostic tools for detection of different *formae speciales* of *F. oxysporum*, some knowledge was first obtained concerning the genetic diversity of these strains. Specifically, do molecular characteristics correspond to the host specialization of the fungus and do strains with a given host specificity form a single natural collection of isolates? If a *forma specialis* corresponds to a single monophyletic, asexually reproducing lineage, it may be reasonable to expect that a single genetic locus can be identified that is diagnostic for that lineage / *forma specialis*. However, if several distant lineages of *F. oxysporum* have the same host specificity, no single locus may correspond to *forma specialis*. In the latter case, several informative markers may be required in order to correspond to the individual lineages within a *forma specialis*. These different lineages may also have different ecological or pathogenic properties that must be tested.

During the period of funding, we tested isolates of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* for monophyletic origin (O'Donnell et al. 1998). Six strains of *F. oxysporum* f. sp. *lycopersici* and four strains of *F. oxysporum* f. sp. *radicis-lycopersici* were selected to represent a range of genetic diversity found in our earlier study of a worldwide collection of these pathogens (Rosewich, et al., 1997). Thirty-seven additional strains of *F. oxysporum* representing 10 other *forma speciales* as well as nonpathogenic strains were also included in the study. Outgroups were *Fusarium* species considered putative sister taxa to the *F. oxysporum* complex. Several independent gene sequences were obtained including those for the nuclear-encoded β -tubulin, calmodulin and translation elongation factor EF-1 α , and the mitochondrial gene for the small subunit ribosomal RNA (mtSSU). Initially, DNA sequences from each locus were analyzed individually by parsimony using the computer program PAUP (Swofford, D.L. 1997. Phylogenetic Analysis Using Parsimony 4.0d59 beta test version. Sinauer, Sunderland, MA). The results of a partition homogeneity test indicated concordance of data sets so they were combined to yield a single most parsimonious tree (Figure 1) in which strains of *F. oxysporum* f. spp. *lycopersici* and *radicis-lycopersici* did not form monophyletic groups. Strains representing different *F. oxysporum* f. spp. *lycopersici* and *radicis-lycopersici* clonal lineages were more closely related to non-pathogens or pathogens of other hosts than they were to each other.

Monophyly of *F. oxysporum* f. spp. *lycopersici* and *radicis-lycopersici* was further evaluated using a log likelihood test. The null hypothesis was that each *forma specialis* was monophyletic. Parsimony trees were then constructed using *forma specialis* monophyly as a constraint and compared with the unconstrained most parsimonious tree. Tree lengths with

enforced monophyly were anywhere from seven (for *F. oxysporum* f. sp. *radicis-lycopersici*) or 12 steps longer (for *F. oxysporum* f. sp. *lycopersici*) than the most parsimonious tree. The null hypothesis that there was no difference between the trees was strongly rejected for each monophyly constraint. This is the strongest evidence to date that the *formae speciales* of *F. oxysporum* from tomato are not monophyletic.

Based on this and other published and soon to be published (Rosewich, et al., 1999 a&b) population genetic studies, we conclude that *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* contain, at a minimum, three and four separate origins, respectively. For *F. oxysporum* f. sp. *lycopersici*, lineages correspond to 1) VCG 0030 and 0032, 2) VCG 0031, and 3) VCG 0033. A fourth lineage of *F. oxysporum* f. sp. *lycopersici* may also exist but only a single race 1 historical isolate (>30 years old) from Italy has been identified from that lineage (Rosewich et al., unpublished). The four *F. oxysporum* f. sp. *radicis-lycopersici* lineages correspond to 1) VCG 0092 and 0096, 2) VCG 0091, 3) VCG 0094 and 0098 and 4) VCG 0099 (Katan et al. 1991; Rosewich et al. 1999 a&b). Other, relatively minor and geographically restricted lineages of *F. oxysporum* f. sp. *radicis-lycopersici* also seem to exist.

Since the genetic diversity we have observed within a *forma specialis* has been high and since, in some instances, isolates in different *formae speciales* may be more similar than isolates from the same *forma specialis*, we have examined the *F. oxysporum* from tomato for other ecological and pathogenic properties. One surprising result was that inoculum of *F. oxysporum* f. sp. *lycopersici* could be spread aerially (Katan, et al., 1996, 1997). Aerial dispersal of conidia of *F. oxysporum* f. sp. *radicis-lycopersici* has been known for some time, so the finding that *F. oxysporum* f. sp. *lycopersici* also could be spread in this manner serves to emphasize the

similarities between the diseases caused by the two pathogens. Likewise it has been observed that approximately 20% of isolates from tomato plants expressing crown rot symptoms are actually *F. oxysporum* f. sp. *lycopersici* based on RFLPs, VCG analysis and pathogenicity testing (Harveson et al., 1997, 1998; Rosewich et al., 1999 a&b).

VCG testing and RFLP analysis also have revealed variation within *formae speciales* of the fungus (Katan and Katan, 1999, Rosewich, et al., 1999 a&b). Nine vegetative compatibility groups of *F. oxysporum* f. sp. *radicis-lycopersici* have been described among hundreds of isolates obtained from 10 countries (Katan, 1999, Katan and Katan, 1999). Prior to our investigations, isolates of *F. oxysporum* f. sp. *radicis-lycopersici* had not been examined for genetic diversity. Several hundred *F. oxysporum* samples from tomato plants displaying crown rot symptoms were collected in central and southern Florida and analyzed using VCGs and RFLP data. VCG 0094, previously reported only from northwestern Europe, was predominant in the collection. A comparison was made between Florida populations of VCG 0094 and VCG 0094 isolates from the Netherlands, Belgium and the United Kingdom. Population subdivision was evaluated by analysis of molecular variance (AMOVA). Comparisons showed no significant difference between the European population and the population from Palm Beach County, Florida. Gene diversity among Florida isolates of 0094 was more than five times greater than the European population. Haplotypes for samples from the three European countries were essentially a subset of those found in Palm Beach County. These results strongly support the inference that the European population resulted from inter-continental migration of isolates from Florida. Two additional, newly described VCGs of the crown rot pathogen (0098 and 0099) were detected, each in five counties in Florida.

Description of cooperation

Since the initiation of their first joint project in 1990, Dr. Kistler and Dr. Katan have been cooperating continuously in research on phytopathogenic *Fusarium oxysporum*. This cooperation is based on their complementary specializations: Dr. Kistler in developing molecular markers and genome analysis, and Dr. Katan in vegetative compatibility and population genetics of various *F. oxysporum* pathosystems on vegetable, ornamental and field crops. The cooperation also involved junior members: S. Kalman (graduate student in Katan's lab) visited UF in 1991, and Dr. E. Boehm (postdoc in Kistler's lab) spent six months as a BARD postdoc Fellow at ARO in 1995. Besides joint publications, this long-lasting cooperation has resulted in professional activities beyond the binational level, i.e. establishing an international *Fusarium* working group, coordinating the international *Fusarium* VCG-numbering system (endorsed by International Society for Plant Pathology), creating (jointly with Culture Collections in the US and Europe) VCG-oriented reference culture collection of *F. oxysporum*, and organizing periodic international *Fusarium* workshops. This proposal allowed for unique opportunity for combining the specific expertises of the co-investigators to study the widely diverse *Fusarium*/tomato pathosystems in Florida and Israel, that jointly represent major parts of *F. oxysporum* f. sp. *lycopersici* and the entire genetic spectrum of *F. oxysporum* f. sp. *radicis-lycopersici* recognized to date.

Overall we have been very pleased with the extent of the cooperation. Dr. Talma Katan visited the Plant Pathology Department at University of Florida from August 22 to August 26, 1995. On August 25, 1995 she presented a seminar entitled "Genetic diversity in the *Fusarium* pathogens from tomato" to the faculty and students of the plant pathology department. During

the visit, Drs. Katan and Kistler planned future research and experiments. During the week of March 18, 1997, Drs. Katan and Kistler met at the 19th Fungal Genetics Conference in Pacific Grove, California in order to discuss research plans and to give a joint poster presentation entitled: "Morphological and molecular characterization of isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* from Florida." Drs. Katan and Kistler also co-organized a workshop on the fungal genus *Fusarium* for the Sixth International Mycological Congress held August 1998 in Jerusalem. Further contact between Dr. Katan and Dr. Kistler occurred at the 20th Fungal Genetics Conference, held March 23-28, 1999 at Pacific Grove, California where current research, joint publications and coordination of stock culture storage was discussed.

The investigators have exchanged numerous cultures and clones. Dr. Katan has sent to UF, approximately 20 standard isolates representing the various VCGs identified for *F. oxysporum* f. sp. *radicis-lycopersici* (VCGs 0090 I, 0090 II, 0090 III, 0091 I, 0091 II, 0092, 0094) and for *F. oxysporum* f. sp. *lycopersici* (VCGs 0030 and 0031). Additionally, clones of random single-copy sequences were provided by Dr. Katan to Dr. Kistler and a probe for DNA fingerprinting provided by Dr. Kistler to Dr. Katan. Dr. Kistler has provided isolates of *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici* for Dr. Katan so that they may be characterized for VCG.

Evaluation of the research achievement with regard to the original proposal and objectives

Our original objectives were to find DNA segments in *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici* that were specific to these pathogens, to localize them to chromosomes and to determine their DNA sequence. While we have accomplished these goals

(Rosewich et al., 1999a&b; Rosewich, et al., manuscript in preparation), the emphasis of our research changed as it became apparent that the *formae speciales* were more genetically complex than anticipated. Thus a few changes in research direction were made from those included in the original proposal. In response to reviewers of the proposal, initially more emphasis was placed on characterization of unique DNA sequences for the purpose of developing diagnostic probes. While at first this seemed to be a readily achievable goal, in the past two years we have confirmed that the *F. oxysporum* tomato pathogens are composed of at least seven major clonal lineages and likely numerous other minor lineages. Therefore, no single probe likely will be diagnostic for “*F. oxysporum* f. sp. *radicis-lycopersici*” or “*F. oxysporum* f. sp. *lycopersici*.”

Conclusions

Several of the most important findings of this research could not have been anticipated when the proposal was written but were discovered only after work had begun on the project. A surprising result was the high level of diversity among isolates of a given *forma specialis*, the similarity between some isolates in different *formae speciales* and the conclusion that various lineages within a *forma specialis* likely had separate evolutionary origins. Each one of these observations changes our way of thinking about the pathogens and their evolutionary origin (Kistler, 1997, 1999). Also striking was our observation that these pathogens, while considered genotypically stable due to their asexual reproductive life history, are actually changing rapidly at some genetic loci (Rosewich, et al., 1999c). Genetic diversity studies also have led to our ability to detect and monitor population changes of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* in the field and investigate local and inter-continental movement of the pathogens.

Perhaps the most lasting contribution of this research is that we have developed the molecular tools to make it possible to study the population genetics and, ultimately, the epidemiology of these two very important fungal pathogen of tomato.

List of Publications

Katan, T., E. Shlevin, and J. Katan. 1996. Airborne inoculum of *Fusarium oxysporum* f. sp. *lycopersici* in greenhouses of cherry tomatoes. *Phytoparasitica* 24: 143 (Abstr.).

Harveson, R.M., **H.C. Kistler**, **T. Katan** and J. Scott. 1997. Evaluation of tomato lines for resistance to fusarium crown rot. *Phytopathology* 87:S40. (Abstr.).

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Kistler, H.C. 1997. Genetic diversity in the plant pathogenic fungus, *Fusarium oxysporum*. *Phytopathology*. 87:474-479.

Rosewich, U.L., R.E. Pettway, **H. C. Kistler** and **T. Katan**. 1997. Morphological and molecular characterization of isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* from Florida. Abstract. Proceedings of the 19th Fungal Genetics Conference, Pacific Grove, California. March 18 - 23, 1997.

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Katan, T. and J. Katan. 1999. Vegetative compatibility grouping in *Fusarium oxysporum* f. sp. *radicis-lycopersici* from the U.K., the Netherlands, Belgium and France. *Plant Pathology*. In press.

Kistler, H.C. 1999. Evolution of host range in *Fusarium oxysporum*. in B. Summerell, ed. *Proceedings of the Paul Nelson Memorial Symposium*. APS Press, St. Paul, MN. In press.

Rosewich, U.L., R.E. Pettway, **H.C. Kistler** and **T. Katan**. 1999a. Population genetic analysis of a lineage of *Fusarium oxysporum* f. sp. *radicis-lycopersici* found in Florida and northwestern Europe. *Phytoparasitica* 27: 72 (Abstr.).

Rosewich, U.L., R.E. Pettway, **T. Katan**, and **H.C. Kistler**. 1999b. Population genetic analysis corroborates the dispersal of *Fusarium oxysporum* f. sp. *radicis-lycopersici* from Florida to Europe. *Phytopathology*. In press.

Rosewich, U.L., R.E. Pettway, and **H.C. Kistler** 1999c. *Joyrider*, a hypervariable element from *Fusarium oxysporum*. APS/CPS Annual Meeting. Submitted abstract.

Rosewich, U.L., R.E. Pettway, **T. Katan**, and **H.C. Kistler**. 2000. Large changes have recently occurred in populations of the tomato wilt pathogen present in Florida. Manuscript in preparation.