Invited Trends Article

Molecular Genetic Approaches to the Study of Phytopathogenic Fungi

Todd C. Peterson

Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-5000 Köln 30, Bundesrepublik Deutschland

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The broad application of chemical crop protectants and intensive plant breeding for the development of agriculture and the management of plant disease have created conditions of environmental concern. Molecular genetic methods are being applied to the analysis of phytopathogenic fungi and their corresponding diseases. Rapid development of this research field will permit the isolation and characterization of genes involved in pathogenic processes. A detailed understanding of the molecular events of disease onset and progression will facilitate the design of improved procedures for disease prevention and control.

Many of the environmental problems of great concern today are either directly or indirectly related to past and present agricultural practices. The challenge to provide sufficient food for a growing human population has largely failed to adequately consider the ecological effects of crop plant breeding and distribution, prolonged use of chemical fertilizers, and widespread employment of chemical agents to control plant disease. To address these issues, research to analyze the ecological impact of these practices is needed. Another essential approach is to investigate the interactions between crop plants and plant pathogens. This article focuses upon the application of molecular genetics to study phytopathogenic fungi. This approach promises to greatly enhance our understanding of many diseases caused by phytopathogenic fungi, and to direct the development of new strategies for improving various properties of crop plants and controlling plant disease.

The phytopathogenic fungi are a diverse collection of parasitic organisms that cause plant diseases and thus a substantial reduction of crop yields worldwide. Despite extensive crop breeding programs to introduce traits for fungal resistance into many agriculturally important crop species and widespread use of fungicides, the economic and agricultural impact of diseases caused by phytopathogenic fungi remains staggering.

Reprint requests to Todd C. Peterson.

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Broad and fundamental knowledge and understanding of the interactions between pathogen and host involved in disease onset and progression are required to direct modern crop plant breeding and fungal disease control efforts of prevention, containment, and eradication. To investigate the processes involved in phytopathogenicity, early workers utilized cytological and biochemical approaches. While this work provided a strong foundation of basic knowledge concerning the general structural and biochemical aspects of fungal disease, the information from these studies is largely descriptive. In addition, population and classical genetic methods were used to identify loci involved in fungal pathogenesis and host resistance. These studies fostered the proposal of models [1, 2] for the interaction between pathogen and host which are generally tenable today.

Unfortunately, many important fungal pathogens are not readily amenable to classical genetic analysis. In addition, phytopathogenic fungi are sufficiently diverse in their life cycles, geographical distribution, and parasitic strategies that the general utility of models derived from genetic analysis of particular species may be limited. Finally, such models, while of conceptual value, are of restricted practical utility as they are unable to provide detailed information concerning the molecular nature and activity of the loci and gene products they describe.

The emergence of recombinant DNA technology and its associated methods has provided the tools necessary to analyze the complexities of pathogen/plant interactions at the molecular level. Among the important areas to be investigated are: 1) the

molecular properties that render particular fungal species pathogenic upon particular host plants or host plant varieties; 2) the events of molecular recognition and action that lead to fungal pathogenesis or host resistance and; 3) the ways this knowledge can be applied to develop procedures for prevention and control of disease.

Molecular Genetic Systems – Development and Utility

A molecular genetic approach to the study of phytopathogenic fungi provides several obvious advantages. Due to experimental difficulties associated with performing and scoring sexual genetic crosses, many important fungal pathogens can not be analyzed by conventional genetic methods. In some species, the sexual cycle is not described or rarely observed. For example, using the molecular method of electrophoretic karyotyping [3], genes can be mapped to specific chromosomes in the absence of a sexual genetic system. In addition, despite the apparently important role of parasexual mechanisms [4, 5] in generating genetic diversity that is responsible for the rapid adaptability of many species, information concerning the molecular basis for such processes remains sparse and elusive using conventional approaches. Molecular genetic methods permit one to devise strategies to isolate and characterize the genes involved in pathogenesis for which there is a simple genetic basis and a selectable or screenable phenotype. Lastly, genetic proof that particular genes, implicated in pathogenesis by any number of criteria, are responsible for specific pathogenicity phenotypes can be obtained by reintroducing such genes into suitable recipient species or strains and observing the expression of the corresponding phenotype(s). Thus, the development of a DNA transformation system to facilitate transfer and expression of genes involved in phytopathogenesis is essential and central to molecular genetic analysis.

Within the past several years, transformation procedures have been established for a continually expanding list of important phytopathogens. Successful transformation procedures generally consist of several common essential features pertaining to preparation of fungal cells, treatment of cells to facilitate the uptake of exogenous DNA, regeneration of fungal cells into mycelia after treatment, and choice of the appropriate transformation vectors and selective systems (see [6] for review). As vegetative hyphae and spores are walled structures, procedures must be developed to isolate wall-less fungal cells, usually as protoplasts, to permit the uptake of DNA. For some obligate biotrophic fungi that can not be manipulated *in vitro,* young germlings may be treated with alkali ions [7] or electroporated [8] to induce DNA uptake. The generation of protoplasts is usually accomplished by treating young mycelia or germinated spores with commercially available mixtures of wall-degrading enzymes in an osmotic buffer to prevent protoplast lysis. Treatment of fungal protoplasts with polyethylene glycol in the presence of calcium ions or electroporation are commonly used to facilitate the entry of DNA into the cells. Lastly, a method for efficient regeneration of treated protoplasts into viable mycelia is required.

Selection Systems and Transformation Vectors

Various dominant selectable genes and their corresponding selective agents are currently available for use in fungal transformation. This type of selectable gene allows the isolation of transformants without prior production and characterization of appropriate auxotrophic mutants. The dominant selectable genes used for fungal transformation generally fall into several classes, either prokaryotic derived genes for resistance to antibiotics [9], or fungal genes which confer resistance to fungicides such as benomyl [10], or markers that permit fungal growth under particular conditions of nutrient limitation [11]. When establishing a transformation system, it is necessary to empirically determine the particular selective system and conditions that produces the best results from among those available as the level of cytotoxicity and frequency of variants or spontaneous mutants resistant to a given selective agent varies among different species.

Transformation vectors contain one of the dominant selectable markers mentioned above under the control of a promoter sequence that facilitates its expression in the fungus to be transformed. In addition, transformation vectors contain a selectable gene and origin of replication to allow propagation of the vector in a bacterial host. The choice of the promoter sequence is important to achieve successful transformation. *A priori,* one should select a promoter from a fungal gene which is likely to be expressed at high levels. Furthermore, if the vector is

to be generally useful for establishing fungal transformation in a broad range of species, the promoter should be derived from a well conserved metabolic gene such as glyceraldehyde-3-phosphate dehydrogenase [12], from a constitutively expressed structural gene such as actin or tubulin [13], or from a gene involved in a universally conserved stress response such as *Hsp 70* [14]. In some cases, homologous functional promoters have been isolated from a "promoter trap" library by *in vivo* selection for expression of the linked antibiotic resistance gene after transformation [15].

Following the isolation of transformed, regenerated fungal colonies, the physical state of the transforming DNA can be analyzed by genomic DNA blotting. Transformation of phytopathogenic fungi occurs primarily *via* integration of the transforming DNA into fungal chromosomes, and cases of homologous integration have been reported [15]. Recently, an autonomously replicating transformation vector has been described [16]. Homologous integration may permit targeting of gene disruption events [17] to construct mutations at loci with uncharacterized functions [18]. Many factors, including the form of vector DNA and the length and degree of sequence homology, may influence the frequency and nature of integration events.

The species utility range of transformation vectors correlates generally with phylogenetic relationship, although exceptions are known. Among higher fungi, the ascomycetes represent the majority of phytopathogenic species for which transformation procedures are established. Several vectors containing promoters with the features described above have widespread utility [19]. For the basidiomycetes, this correlation is also observed, although fewer species have been analyzed. The oomycetes, lower fungi more distantly related to the fungi above, include several important plant pathogen genera, for example *Phytophthora* and *Bremia*. Transformation of these fungi using the vectors developed for the higher fungi above have not been successful. This indicates that transformation of these species will probably require the construction of vectors which contain selectable markers under the control of homologous promoters. The reported transformation of *Achlya*, a nonpathogenic aquatic oomycete using a vector containing an animal virus SV40 promoter controlling the kanamycin resistance gene [20] appears to be an exceptional case as attempts by several groups to transform other oomycetes using vectors containing diverse eukaryotic and prokaryotic promoters have not been successful. One direct method for obtaining promoters for homologous vector development entails the isolation of specific, well conserved genes from genomic libraries by heterologous hybridization. The genes encoding ubiquitin, actin, tubulin, or *Hsp 70* represent excellent candidates for this approach.

Model Systems and Future Aspects

Several pathogen/host systems demonstrate the utility and potential of a molecular genetic approach. The analysis of the regulation and expression of the cutinase gene indicates the pivotal role it plays in a primary critical step in pathogenesis, fungal penetration of the outermost barrier of the host, the cuticle. Cutinase is secreted in large amounts at the tips of penetrating hyphae. The cutinase gene of *Fusarium solani* f.sp. *pisi* is induced specifically by cutin monomers, the products of cutin hydrolysis. Cutinase monomers bind specifically to a nuclear protein factor to form a stable complex. Formation of this complex results in the phosphorylation of a second protein and cutinase gene induction. Induction-specific binding of nuclear proteins to the 360 bp cutinase minimal promoter is also observed. The molecular mechanism by which these proteins act to induce the cutinase gene is being investigated. The critical role of cutinase in pathogenesis is demonstrated by cutinase gene transfer experiments. Transformation of the *F. solani pisi* cutinase gene into *Mycosphaerella*, a wound pathogen of papaya, renders this fungus pathogenic on intact fruit [21, 22].

Plants produce a variety of protective compounds that are cytotoxic to fungal pathogens. Pathogenicity of *Nectria haematicocca* on pea plants requires the activity of pisatin demethylase, an enzyme that inactivates the host-specific fungal toxin pisatin [23]. The role of this enzyme in pathogenesis is indicated by the observation that the corn pathogen *Cochliobolus heterostrophus* can cause disease symptoms on pea plants after transfer of the *PDA1* gene encoding this enzyme. Other genes encoding pathogenicity functions are also involved in disease progression since the symptoms observed after infection of pea plants with *PDA1*$^+$ *C. heterostrophus* transformants are less severe than for *N. haematococca* infection, and the transfer of *PDA1* to the saprophyte *A. nidulans* does not confer the ability to colonize plants [24].

To a large extent, the field control of phytopathogenic fungi relies upon the use of fungicides. This method of disease control presents several problems. For example, the emergence of field isolates which display increased fungicide resistance is often observed, and increased, alternative or combinatorial use of fungicides often results only in temporary control. Since fungicides generally affect pathogen and non-pathogen species, these practices can radically alter soil ecology causing the expansion of other pests and substantial environmental imbalance. The molecular mechanism for resistance to the fungicide benomyl, originally investigated in the non-pathogens *N. crassa* and *A. nidulans,* is being analyzed in several pathogens including the wheat pathogen *Septoria nodorum* and the brassica pathogen *Leptosphaeria maculans* [25]. An *A. nidulans* benomyl resistance gene probe was used to isolate the *S. nodorum* β-tubulin gene *tubA*^{r} from a benomyl-resistant mutant by heterologous hybridization. The isolated *tubA*^{r} gene transforms *S. nodorum* and *L. maculans* to the benomyl-resistant phenotype. β-Tubulin gene and protein sequence analysis of wild type and benomyl-resistant mutant genes will provide insight into the molecular mechanism of resistance. In principle, transformation may be used as a tool to isolate and characterize other fungicide resistance genes. With detailed information concerning the molecular features of resistance, a framework can be established to direct the development of fungicides with more specific and desirable properties.

These studies represent a set of potential molecular target steps, including regulation of gene expression and enzyme activities involved in pathogenicity, and the molecular basis for fungicide resistance to be further explored. The methods of molecular genetic analysis are becoming generally applicable to phytopathogenic fungi. Other related areas of study pertaining to the mechanisms of rapid adaptability, the analysis of particular physiological aspects, the field distribution of species and specific pathotypes, and the understanding of phylogenetic relationships can also be explored using molecular methods. The future holds great promise that the basic knowledge of disease processes gained from molecular genetic analysis of phytopathogenic fungi will be expanded and applied to design improved methods for disease prevention and control.

Note added in proof:

A current and comprehensive review of the topic, S. A. Leong and D. W. Holden, Ann. Rev. Phytopathol. **27,** 463–481 (1989), has recently been published. The work reported in reference [24] has recently been published in W. Schafer, D. Straney, L. Ciuffetti, H. D. Van Etten, and O. C. Yoder, Science **246,** 247–249 (1989).

[1] H. H. Flor, Ann. Rev. Phytopathol. **9,** 275–296 (1971).
[2] P. R. Day, Genetics of Host-Parasite Interactions, W. H. Freeman and Co. 1974.
[3] P. L. Skatrud and S. W. Queener, Gene **78,** 331–338 (1989).
[4] R. D. Tinline and B. H. MacNeill, Ann. Rev. Phytopathol. **7,** 147–170 (1969).
[5] W. J. Tolmsoff, Ann. Rev. Phytopathol. **21,** 317–340 (1983).
[6] S. A. Leong, Adv. Plant Pathol. **6,** 1–26 (1988).
[7] M. B. Dickman, Curr. Genet. **14,** 241–246 (1988).
[8] I. Karube, E. Tamiya, and H. Matsuoka, FEBS Lett. **182,** 90–94 (1985).
[9] K. R. Kaster, S. G. Burgett, and T. D. Ignolia, Curr. Genet. **8,** 353–358 (1984).
[10] M. Orbach, B. Porro, and C. Yanofsky, Mol. Cell Biol. **6,** 2452–2461 (1986).
[11] B. G. Turgeon, R. C. Garber, and O. C. Yoder, Mol. Gen. Genet. **201,** 450–453 (1985).
[12] A. M. Musti, Z. Zehner, K . A. Bostian, B. M. Paterson, and R. A. Kramer, Gene **25,** 133–143 (1983).
[13] N. A. Neff, J. H. Thomas, P. Grisafi, and D. Botstein, Cell **33,** 211–219 (1983).
[14] J. Wang, D. W. Holden, and S. A. Leong, Proc. Natl. Acad. Sci. U.S.A. **85,** 865–869 (1988).
[15] B. G. Turgeon, R. C. Garber, and O. C. Yoder, Mol. Cell Biol. **7,** 3297–3305 (1987).
[16] T. Tsukuda, S. Carelton, S. Fotheringham, and W. K. Holloman, Mol. Cell Biol. **8,** 3703–3709 (1988).
[17] J. W. Kronstad, J. Wang, S. F. Covert, D. W. Holden, G. L. McKnight, and S. A. Leong, Gene **79,** 97–106 (1989).
[18] I. G. Jones and H. M. Sealy-Lewis, Curr. Genet. **15,** 135–142 (1989).
[19] P. J. Punt, R. P. Oliver, M. A. Dingemanse, P. H. Pouwels, and C. A. M. J. J. van den Hondel, Gene **56,** 117–124 (1987).
[20] E. K. Manavathu, K. Suryanarayana, S. E. Hasnain, M. Leung, Y. F. Lau, and W. C. Leung, Gene **57,** 53–59 (1987).
[21] G. K. Podila, M. B. Dickman, and P. E. Kolattukudy, Science **242,** 922–925 (1988).
[22] G. K. Podila, M. B. Dickman, and P. E. Kolattukudy, Proc. EMBO Workshop Molecular Biology of Filamentous Fungi, Helsinki, Finland 1989 (in press).
[23] K. M. Weltring, B. G. Turgeon, O. C. Yoder, and H. D. Van Etten, Gene **68,** 335–344 (1988)
[24] O. C. Yoder, Proc. EMBO Workshop Molecular Biology of Filamentous Fungi, Helsinki, Finland 1989 (in press).
[25] R. N. Cooley and C. E. Caten, Proc. EMBO Workshop Molecular Biology of Filamentous Fungi, Helsinki, Finland 1989 (in press).