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Unravelling and managing fusiform rust disease: a model approach for coevolved forest tree pathosystems

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Summary

Fusiform rust disease remains the most destructive disease in pine plantations in the southern United States. Our ongoing research is designed to identify, map, and clone the interacting genes of the host and pathogen. Several resistance (R) genes have been identified and genetically mapped using informative pine families and single-spore isolate inoculations. In addition, we are mapping the first of many expected corresponding avirulence (Avr) genes in the fungal pathogen. The Avr genes condition avirulence/virulence and avirulence is required for an incompatible reaction (i.e., no-gall development) to take place within an inoculated tree that carries resistance at the corresponding R gene. We provide an overview of our methodology for identifying and mapping R and Avr genes, an update of our current progress, and a brief discussion of two approaches for predicting R gene genotypes of uncharacterized parental trees and for estimating the efficacy of specific pine genotypes at various planting locations. This paper emphasizes the critical importance of controlled genetic materials of both the host and pathogen for elucidating the genetic nature of resistance and virulence in coevolved forest pathosystems.

1 Gene-for-gene interaction in fusiform rust disease

Foresters have long recognized the presence of fusiform rust disease resistance in pines of the southern United States (Barber et al. 1957). However, until only relatively recently resistance to fusiform rust was thought to be quantitative in nature, or at least tree breeders largely treated it as a quantitative trait (Zobel and Talbert 1984). This is not surprising considering that the relative resistance among pine families was measured as the percentage of diseased progeny obtained when screened with genetically heterogeneous collections (i.e. mixtures of spores from many galls) of the rust fungus. It was not until less heterogeneous pathogen populations were utilized, in the form of single gall collections and single aeciospore isolates (Powers 1980), that a shift towards considering fusiform rust resistance in more qualitative terms began to occur.

Research results reported over the last and current decades (Kinloch and Walkinshaw 1991; Nelson et al. 1993; Wilcox et al. 1996; Amerson et al. 1997, 2004; Kuhlman et al. 1997; Stelzer et al. 1999; Kubisiak et al. 2005; H. V. Amerson, unpublished data) have led us to conclude that gene-for-gene interactions largely determine gall formation in fusiform rust disease (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Cumm.) Burds. & Snow – loblolly pine (*Pinus taeda* L.) and slash pine (*P. elliottii* Engelm. var. *elliottii*) pathosystems). In this paper we present the experimental approach and data analysis that we are using to examine the gene-for-gene nature of the fusiform rust – loblolly pine pathosystem and discuss two strategies for managing against losses to this economically important disease. It is our hope that this overview will serve as a model approach for elucidating the genetic nature of resistance and virulence in other coevolved

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forest pathosystems and thus lead to more efficacious strategies for managing against losses to such diseases.

2 Experimental system and derivation of host and pathogen genotypes

The basic experimental design and typical results for our recent gene-for-gene experiments are depicted in Table 1. The methodology employed is similar to that used in the cereal rust systems, where host-by-pathogen interactions are evaluated as incompatible (I) or compatible (C). Cereal rust researchers refer to these as low (L) or high (H) infection type (or on a scale from 0–4, where 0–3 are L and 4 is H), but we prefer and have adopted I and C in recognition of the interaction between the organisms. Further we note that Loegering (1966) used the terms definitive (1) and non-definitive (0) and coined the term 'aegricorpus' to represent the interaction organism formed by the host and pathogen, while Nance et al. (1992) and Nelson et al. (1993) used the definitive/non-definitive terminology but substituted 'union phenotype' for aegricorpus. Our interaction classifications are initially based on the percentage of seedlings from a loblolly pine family that are galled by a single-spore isolate of *C. quercuum* f. sp. *fusiforme* (Cqf). Currently, high disease levels (≥80% galled) are classified as C and significantly lower disease levels (≤65% galled) are classified as I, while intermediate levels (>65% and <80%) are noted as ambiguous. However, all classifications are considered tentative until the interaction has been subjected to DNA marker evaluations (described in Section 3).

Our interpretation of the hypothetical data in Table 1 is that Isolate A is avirulent to a corresponding resistance gene (R1) that is segregating in pine Family 1. When per cent gall is between about 35% and 65%, as in this example, we infer 1 : 1 segregation indicating that a single dominant R gene (R1 in this case) is segregating and interacting with pathogen avirulence. This implies that one parent is heterozygous 'Rr' at R1 while the other parent is homozygous 'rr'. When open-pollinated families are used, this implies that the female parent is heterozygous and the frequency of the 'R' allele is relatively low to zero in the pollen source. Since haploid basidiospores that arise from dikaryotic (diploid during the telial stage) isolates infect pine we refer to isolate genotypes as being either homozygous or

Table 1. **Typical range in percentage of loblolly pine (*Pinus taeda*) seedlings galled with Cqf (*Cronartium quercuum* f. sp. *fusiforme*) and the host-pathogen classification (I, incompatible; C, compatible).**

	Pathogen lines (Cqf)[2] (% of trees galled, I or C)	
Host lines (pine)[1]	Isolate A	Isolate B
Family 1	35–65, I	80–95, C
Family 2	80–95, C	35–65, I
Family 3	80–95, C	80–95, C
Family 4	35–65, I	35–65, I

Using the analytical approach described in Section 2, the hypothesized host and pathogen genotypes are as follows (R, resistance; r, susceptibility; A, aviulence; a, virulence): Family 1 – R1r1,r2r2; Family 2 – r1r1,R2r2; Family 3 – r1r1,r2r2; Family 4 – R1r1,R2r2; Isolate A – A1A1,a2?2; Isolate B – a1?1,A2A2.

[1]Optimally, host lines are full-sib families where one parent is known to be highly susceptible to Cqf. However, open-pollinated families have also been used routinely, with the female parent being the expected source of resistance. Although families of progeny are challenged, the parental tree carrying resistance is the genotype being characterized.

[2]Cqf isolates should be derived from a single dikaryon, such as a urediniospore collected from the primary oak (*Quercus* spp.) host or an aeciospore collected from a pine gall.

heterozygous at Avr genes. Furthermore, in the case of an incompatible interaction an isolate must be homozygous for avirulence ('AA') to the corresponding R gene because heterozygous ('Aa') isolates cause a compatible interaction when the concentrated basidiospore inoculation system of Matthews and Rowan (1972) is employed (Kubisiak et al. 2005).

Continuing with Table 1, Isolate B is avirulent to the resistance gene R2 that is segregating in Family 2, but virulent to gene R1 segregating in Family 1. It is also relevant to note that Isolate A is virulent to the R gene (R2) segregating in Family 2. Family 1 is segregating for resistance at R1 but is not segregating at R2, i.e., all Family 1 progeny are susceptible ('rr') at R2. Conversely, Family 2 is segregating for resistance at R2 but is not segregating at R1. Family 3 does not segregate for resistance at R1 or R2 and Family 4 is likely to segregate for resistance at both R1 and R2. However, two other possibilities exist: Family 4 could be segregating for resistance at a third R gene (R3), with both Isolates A and B avirulent at the corresponding gene; or Family 4 could be segregating for resistance at two different R genes (R3 and R4), with Isolates A and B avirulent for one or the other corresponding gene, respectively. When cases are encountered where multiple scenarios could explain the data, we apply the principle of Occam's razor (i.e. the simplest explanation is preferred, in this case the fewest corresponding gene pairs) until evidence suggests otherwise.

The less than 100% gall formation (e.g., Family 1-Isolate B) often observed in C interactions is thought to be due to various inefficiencies in the inoculation system with these non-galled individuals commonly termed 'escapes'; however in pine open-pollinated families they may represent pollen source resistance. Although escapes have been an object of concern and debate, protocols to minimize their occurrence have been imposed, e.g. the use of full-sib host materials consisting of crosses between trees having shown some resistance and a highly susceptible tree, and the use of high inoculum densities (≥100 000 basidiospores/ml) (Kuhlman et al. 1997). Even though escapes are likely to continue to cause concern, their presence has not created enough experimental error to critically hamper R gene mapping in I interactions (Amerson et al. 1997, 2004).

Although the analysis is simplified when families segregate for only a single R gene (i.e., 'rr' at all other R genes) or isolates are homozygous for avirulence ('AA') at a single Avr gene (i.e., 'Aa' or 'aa' at all other Avr genes), it is not unusual for families and isolates to have more than one interactive (R_ and AA, respectively) gene. In our experience, parent trees that have been useful for R gene mapping tend to be heterozygous for only one or two R genes, whereas isolates are often homozygous for avirulence at multiple Avr genes (H. V. Amerson, unpublished data). Such would be the case for Isolates C, D and E in our expanded example (Table 2). These hypothetical data suggest that Isolate C is homozygous for avirulence to the R genes that are segregating in Families 1 and 2 (R1 and R2, respectively). Further, Family 5 is segregating for resistance at a third R gene (R3) that can be detected by its differential response to Isolates D and E (compared to Isolates A, B and C). We also see that Family 3 is susceptible with respect to all of the R genes investigated in Table 2 and thus could serve as a 'universal suscept' for these three genes. Isolate D is homozygous for avirulence at two Avr genes (Avr2 and Avr3) resulting in I classifications with Families 2, 4 and 5. Finally we note that the Family 4-Isolate C interaction is consistent with the hypothesis that Family 4 is segregating for resistance at two R genes (R1 and R2, as discussed for Table 1) and that Isolate E is homozygous for avirulence with respect to all three R genes. In this way information of the number of corresponding gene pairs (CGPs), and the specific genotypes of parent trees and isolates can be inferred (see Table 2 note).

As suggested above, the most informative parents and isolates are specific for only one CGP and are thus referred to as single-gene differentials (SGDs). It is important to note that SGDs are relative to the CGPs under consideration and may not be SGDs with respect

Table 2. **Host-pathogen interaction classification (I or C) for pine families and fungal isolates in Table 1 plus results for additional isolates C, D, and E and Family 5.**

	Pathogen lines (I or C)				
Host lines	Isolate A	Isolate B	Isolate C	Isolate D	Isolate E
Family 1	I	C	I	C	I
Family 2	C	I	I	I	I
Family 3	C	C	C	C	C
Family 4	I	I	I+[1]	I	I+
Family 5	C	C	C	I	I

Using the analytical approach described in Section 2, the hypothesized pine genotypes are as follows (R, resistance; r, susceptibility; A, aviulence; a, virulence): Family 1 – R1r1,r2r2,r3r3; Family 2 – r1r1,R2r2,r3r3; Family 3 – r1r1,r2r2,r3r3; Family 4 – R1r1,R2r2,r3r3; Family 5 – r1r1,r2r2,R3r3. The hypothesized fungal genotypes are as follows: Isolate A – A1A1,a2?2,a3?3; Isolate B – a1?1,A2A2,a3?3; Isolate C – A1A1,A2A2,a3?3; Isolate D – a1?1,A2A2,A3A3; Isolate E – A1A1,A2A2,A3A3.

[1]Interactions are classified as I+ when percent gall is below 35%, and they are indicative of more than one interacting corresponding gene pair (CGP).

to the whole pathosystem. Single-gene differentials have been difficult to find, especially for isolates, but we can now consider developing them by crossing isolates of known avirulence/virulence composition and selecting on known resistant host genotypes. For example, to convert Isolate D (Table 2) to a SGD for R3 one would proceed as follows. Cross Isolate D with Isolate A, collect D × A aeciospores and inoculate oaks to produce basidiospores. With these basidiospores, inoculate Family 4 and collect aeciospores from isolated galls formed on DNA marker-identified (see Section 3) resistant (R1r1,R2r2,r3r3) trees only (this would fix the alleles at Avr1 and Avr2 as only spores with virulence alleles at both Avr1 and Avr2 can incite gall formation on the R1r1,R2r2,r3r3 trees). Using these aeciospores, inoculate oaks and develop several single-uredinial pustule (SUP) lines (Kubisiak et al. in press; Zambino et al. 2000). Inoculate Family 5 with basidiospores derived from the individual SUP lines, noting the lines that do not incite galls on R3 trees. These lines should be homozygous for avirulence at only a single Avr gene, Avr3, and thus would serve as a SGD for R3. In addition, we recommend that these SUP lines be genotyped with DNA markers to verify their ancestry and purity.

Much progress in recent years makes this approach possible, including the development of Cqf-specific microsatellite DNA markers (Kubisiak et al. 2004; Burdine et al. 2007), the development of the SUP technique (Kubisiak et al. in press) and the identification of DNA markers in pine families that are closely linked to R genes (Wilcox et al. 1996; Amerson et al. 1997, 2004). Ultimately, having access to cloned pine genotypes that are SGDs, as recommended by Nance et al. (1992), would be very useful as this would eliminate the need to produce new differentials for each study using specific families and R gene-linked markers. Progress on pine somatic embryogenesis and cryopreservation now makes host cloning feasible (S. Merkle, University of Georgia, Athens, GA, USA, personal communication).

3 Mapping genes involved in the gene-for-gene interaction

Fusiform rust disease resistance genes have been mapped in loblolly pine using several inoculation matrix experiments as illustrated in Tables 1 and 2 (Wilcox et al. 1996; Amerson et al. 1997, 2004). In any one experiment only a proportion of the cells (family-by-isolate combinations) are potentially informative (I classifications) with respect to

mapping R genes. In these cells, with respect to the R gene, galled trees expectedly are homozygous for the susceptibility allele ('rr'), while non-galled trees are heterozygous ('Rr', where resistance is dominant to susceptibility). Scoring a large number of DNA markers allows for tests of statistical association to be made between the markers and the gall/no-gall disease phenotype. Markers significantly associated with the phenotype are inferred to be linked to the gene (trait locus) conferring the phenotype, in this case the R gene. The degree of association (closeness of linkage) among several markers and the trait locus provides a genetic map of the chromosomal region containing the R gene. Given that we typically genotype megagametophyte DNA (the maternally inherited portion of the progeny's DNA), this map is specific to the maternal parent. Once an R gene has been mapped for a given parent the linked markers are used to determine if I interactions involving this parent's progeny and a different isolate(s) is likely due to the same gene (it maps to the same locus) or to a different gene. However, given our current genetic map resolution we are not able to distinguish among tightly linked R genes whose alleles are linked in coupling phase. In some cases R genes appear to be the same based on interaction phenotypes but map to different loci, which indicates that they are different genes–highlighting the necessity of validating R genes with genetic mapping. Within a family, a matrix cell classified as C would expectedly show non-significant marker-phenotype associations for markers previously shown to be linked to the subject R gene. Also within a family, a cell initially noted as ambiguous but showing significant marker-phenotype associations for markers known to be linked to the subject R gene would be classified as I, while non-significant marker-phenotype associations would result in a C classification. To date we have used random amplified polymorphic DNA (RAPD) (Myburg et al. 2006) and to a much lesser extent amplified fragment length polymorphism (AFLP) (Myburg et al. 2001 modified for pine following Remington et al. 1999) markers for detecting and mapping R genes. In the future we expect to integrate microsatellite DNA markers as increasing numbers are becoming available for loblolly pine (Auckland et al. 2002; Nelson et al. 2007; Echt et al. 2008).

To further verify the gene-for-gene model, we set out to map a corresponding Avr gene in the fungal pathogen. This has proven to be less straight-forward than mapping R genes, but we now have an experiment well underway aimed at mapping *Avr*1 (*Avirulence to Fusiform rust resistance gene* 1, Kubisiak et al. 2005), the corresponding gene to *Fr*1 (Wilcox et al. 1996). Two isolates expected to be homozygous for avirulence and virulence toward *Fr*1, respectively, were crossed by transferring pycniospore drops between galls (after isolating the galls to prevent unintended crossing) and collecting the resulting aeciospores. The aeciospore collections were genotyped for the presence of microsatellite marker alleles diagnostic of the two parental isolates (Kubisiak et al. 2005). Collections showing clear evidence for a cross between the two parents were used to develop a sample of SUP lines. These lines were tested with microsatellite markers to verify their purity and one SUP line was selected and used to produce basidiospores for inoculation of a large full-sib family segregating at *Fr*1. As expected for an isolate heterozygous at *Avr*1 (Kubisiak et al. 2005), nearly all (>95%) of the pine seedlings were galled. Each seedling was genotyped with diagnostic DNA makers to determine its *Fr*1 genotype, and single pycnial drops were collected individually from 'Rr' and 'rr' trees. As shown by Kubisiak et al. (2005), spores carrying either a virulence or an avirulence allele towards *Fr*1 can incite gall formation on 'rr' trees, while only spores carrying a virulence allele can incite gall formation on 'Rr' trees. This differential interaction should be detectable by DNA markers in the fungus that are linked to the corresponding Avr gene, *Avr*1. In addition, a complete genetic map using all segregating markers can be developed using single genotype pycniospore DNA samples from 'rr' trees. At present a large number of RAPD (Doudrick et al. 1993), microsatellite (Kubisiak et al. 2004; Burdine et al. 2007), and AFLP markers

(Anderson et al. 2008) are being screened against Cqf spore samples collected from 'Rr' and 'rr' trees and several appear to be linked to *Avr*1.

4 Using differentials and DNA markers to manage fusiform rust disease

We are beginning to obtain a better understanding of the underlying genetic interactions regarding gall formation in fusiform rust disease. Ultimately our goal is to be able to predict the likely resistance (incidence of gall formation) of various families when planted under field conditions. We hypothesize that if we can predict the frequency of virulence alleles for known Avr genes at various planting locations then tree breeders and forest managers can use this information to select and plant families with effective R genes. The Avr genes with the lowest frequency of virulence would be given priority for selecting pine families with resistance alleles at the corresponding R genes. We can envision two systems for generating the data to make these predictions. One involves the development and use of differential host and pathogen lines. The other involves the development and use of tightly linked and thus highly diagnostic DNA markers in the host and pathogen.

A differential line system will require the identification and/or breeding of single-gene host and pathogen differentials (SGDs). On the host side, once recognized, the differentials should be cloned and preserved for long-term use. A somatic embryogenesis system combined with cyropreservation is the most likely approach, but this could be costly and difficult to maintain over time. Another approach is to select parents that are homozygous ('RR') for a single resistance gene. Crosses among these parents and a universally susceptible parent ('rr') will produce progeny seedlings that are uniformly differential ('Rr') for the gene carried by the differential ('RR') parent. This might be the best approach for the long-term, as potentially heterozygous SGDs have been identified (H. V. Amerson, unpublished data) that can be crossed to produce the single-gene 'RR' and 'rr' parents. On the pathogen side, a crossing scheme could be devised to convert isolates into SGDs as described in Section 2. In this way a set of SGD families and a corresponding set of SGD isolates could be developed for use in monitoring and predicting either Avr gene frequencies in Cqf samples or R gene genotypes in candidates for selection in loblolly pine breeding programs. This approach is also attractive given the well established, cost-effective methods used at the Resistance Screening Centre (U.S. Forest Service, Asheville, NC) and their ability to accommodate single-spore-derived isolates (Kubisiak et al. 2005; Bronson 2008).

A DNA marker system will require markers that are fully diagnostic of specific R genes in the host and Avr genes in the pathogen. In outbreeding species, such as pines and rusts, this is a very challenging proposition because linked loci (marker and trait) tend to be in linkage equilibrium, meaning that specific marker alleles are not diagnostic (i.e., they are independent) of specific trait alleles in natural populations. This can be overcome by studying markers within specific families and isolates, as is currently done in our R gene and Avr gene mapping studies, or by finding very tightly linked markers such as those that are within the actual gene of interest. The latter result requires high-resolution genetic mapping using either very large family-by-isolate sample sizes (>300) or a large samples from random mating populations (>1000 trees, as in mapping by association, see Neale and Savolainen 2004) and a very large number of markers distributed across the genome (Hirschorn and Daly 2005) or targeted for the genome regions that carry clusters of R or Avr genes (Tabor et al. 2002; Liu and Ekramoddoullah 2007). With very tightly linked markers a more direct approach, map-based cloning (e.g., Martin et al. 1993; Haen et al. 2004), can be contemplated. We are still a long way from map-based cloning in the host, given the relatively modest density of genetic markers and the very large physical genome size. However, this endeavour is now plausible on the pathogen side (Kubisiak et al. in press). We are currently positioned to use markers linked to *Avr*1 to identify large insert

genomic DNA clones and construct a physical map of the *Avr*1-containing region. Once a physical map of the region is available we will develop new DNA sequence-based markers and narrow down the region likely to contain candidate genes. This process will be repeated until it is clear that we have the gene, *Avr*1, and we can identify the specific DNA sequence difference(s) between its avirulence and virulence alleles. With this information, allele-specific markers can be developed and used to directly genotype any sample of Cqf.

Clearly much work needs to be done, but our goal remains the same – to be able to reliably predict R and Avr gene status for any loblolly pine parent tree or Cqf spore sample, respectively. The development, maintenance, and continued use of specific differential host families and single-spore pathogen isolates are critical to advancing our understanding of this pathosystem. The implications of correctly interpreting the pathosystem are critical both for tree breeding and seedling deployment and for understanding host-pathogen evolutionary dynamics. To more effectively deploy resistance, a better understanding of the R gene composition of elite parent trees and the geographical distribution of Avr gene frequencies in the pathogen population is needed. For example, progenies derived from elite parents with uncharacterized resistance are likely to experience high disease levels when planted in areas where pathogen virulence is present. Similarly, unacceptable disease levels are possible when resistance characterized progenies are planted in virulence unknown areas. In addition, it will be necessary to know the R gene status of parent trees for the purposes of combining or pyramiding R genes in advanced generation selections and their progeny. We currently do not understand what effect the deployment of specific R genes in commercial plantations will have on the evolution of virulence in Cqf, however, given the large extent of non-commercial pine forests in the southeastern U.S it would seem to be minimal. Until this is well understood, periodic monitoring of Avr genes will be needed to detect increases in virulence allele frequencies well before disease incidence increases to unacceptable levels.

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