GENETIC ARCHITECTURE OF FUNGAL DISEASE TRAITS IN LOBLOLLY PINE

By

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Dedicated to my family and my cats

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In the southeastern United States, loblolly pine (*Pinus taeda* L) is the most common tree species covering nearly 13.4 hectares in southern United States with over 1 billion seedlings produced every year. This popular pine species bring $30 billion and 110,000 jobs to the region. However, two endemic fungal diseases are threatening this productive view: fusiform rust incited by *Cronartium quercuum* Berk. Miyable ex Shirai f. sp. *fusiforme* and pitch canker incited by *Fusarium circinatum* Nirenberg et O'Donnell. Loblolly pine is not totally susceptible to these diseases and it has been shown by many researchers, using natural and artificial inoculations, that loblolly pine families show genetic variation in resistance to both fusiform rust and pitch canker diseases. Precision was acquired by a combination of clonal propagation, which allows repeat observations of the same genotypes and the use of a mixed linear model (GAREML) to adjust for environmental effects. In the first part of this study, I identified traits, clones, families, and parents that guide a genetic approach to dissecting disease traits in loblolly pine. I

verified that pitch canker and fusiform rust traits are heritable and identified the disease traits that are genetically distinct from one another. Second, I used DNA marker information that was developed in previous mapping studies to distinguish host genotypes that carry/lack the pathotype-specific Fr1 allele. I tested the hypothesis that the Fr1 allele is predictive of resistance in greenhouse and field experiments. Because these studies involved clonally propagated materials, I also quantified the extent to which genetic and non-genetic factors influence disease expression levels and escape rate in greenhouse and field trials. Finally, I used gene expression data obtained from a very complex design of microarray experiments using diseased and healthy loblolly pine clones from a family that is segregating for *Fr1*, to identify genes that are differentially regulated in diseased and healthy individuals. I contrasted gene expression in diseased and healthy individuals over a time frame of 4 months. Together, these studies revealed the genetic architecture of fusiform rust disease resistance in scales ranging from the population level to the molecular level.

CHAPTER 1
INTRODUCTION

Forests cover one-third of the earth's terrestrial surface and provide social, economical and environmental benefits (FAO, 2006). Pine is a dominant plant species in Europe, Asia and America and has been used both as source for forest products and as a model organism to study wood formation (Lev-Yadun and Sederoff, 2000). In the southeastern United States, loblolly pine (*Pinus taeda* L) is the most common tree species covering nearly 13.4 hectares in southern United States (Schultz, 1999) with over 1 billion seedlings planted every year (McKeand et al., 2003). This popular pine species brings $30 billion and 110,000 jobs to the region (Schultz, 1999). In addition, loblolly pine plantation and natural forests offer habitat for many diverse species, control erosion, improve water quality, provide recreation and sustain rural communities.

As management of loblolly pine plantations intensive to maximize product yield, new problems started to emerge in production of healthy loblolly pine. Among these problems, two endemic fungal diseases attracted the attention of many researchers and breeders: fusiform rust (incited by *Cronartium quercuum* Berk. Miyable ex Shirai f. sp. fusiforme) (Burdsall and Snow, 1977) and pitch canker (incited by *Fusarium circinatum* Nirenberg et O'Donnell) (Nirenberg and ODonnell, 1998). Loss of millions of dollars (Cubbage et al., 2000) pushed breeders and researchers to investigate fusiform rust, one of the most economically destructive diseases of the southeastern United States. It is incited by *Cronartium quercuum*, a biotrophic pathogen that alternates its life cycle with pine and oak as hosts.

Fusiform shaped galls on pine hosts are the major symptom of fusiform rust. As disease progresses through the years these infections may take the form of sunken cankers. Galls on stems decrease the wood quality and sometimes kill the plant (Schmidt, 1998). Both specific resistance, i.e., "gene-for-gene" interactions (Powers, 1980; Stelzer et al., 1997; Wilcox et al., 1996), and partial resistance in the form of short galls (Schmidt et al., 2000) have been demonstrated for the *C. quercuum*- pine pathosystem. Loblolly pine is not totally susceptible to this disease and it has been shown by many researchers, using natural and artificial inoculations, that loblolly pine families show genetic variation in resistance to fusiform rust (Kuhlman and Powers, 1988; McKeand et al., 1999).

The pitch canker disease is not as economically important as fusiform rust in southeastern United States although it can damage southern pine plantations sporadically in the USA and it is an important problem for *Pinus radiata* in California (Storer et al., 2002). In the southeastern United States seedling production can be severely hampered by this disease (Dwinell et al., 1985). The pitch canker inciting agent, *F. circinatum*, is a necrotrophic fungus that survives on dead tissues. A successful infection results in symptoms like resinous lesions on stems and branches that cause seedling mortality and decreased growth rates and crown die-back of plantation trees (Dwinell et al. 1985). Although genetic variation among loblolly pine families (Kuhlman et al., 1982) and clones (Dwinell and Barrowsbroaddus, 1982) has been detected, the genetic architecture of resistance has not been thoroughly investigated.

Knowing that family level genetic variation for both diseases exists in the same species, namely, loblolly pine, provided an opportunity to investigate and contrast the nature and architecture of resistance to the diseases incited by the biotrophic and

necrotrophic fungi. The main difference between the two types of fungi is that necrotrophs survive on the dead plant cells and biotrophs feed on living plant cells (Lewis, 1973). Thus the damage they cause is significantly different and the host defense mechanisms against them may also vary. For example, biotrophic fungi are typically associated with gene-for gene systems (Glazebrook, 2005; Hammond-Kosack and Jones, 1997) and necrotrophs are often linked to quantitative disease resistance genes (Oliver and Ipcho, 2004). Thus, I hypothesized that resistance and responses on the pathogen and host sides would differ for the two distinct pathosystems.

Complex traits such as disease resistance can be dissected by two core activities, genotyping and phenotyping. These two different sets of data can be analyzed in two ways: by linkage, which uses QTL or linkage analysis approach; or by association, which uses linkage disequilibrium to make genotype-phenotype association (Jannink et al., 2001). Association genetics is gaining favor as an approach to identify genes that underlie complex traits (Rafalski, 2002). The association approach relies on linkage disequilibrium between marker loci and target trait loci, and because many unrelated individuals are examined in a single association experiment, it is possible to evaluate the effects of many alleles across a broad sample of the population (Flint-Garcia et al., 2003). In contrast QTL approaches evaluate pairs of segregating alleles typically within single families (Jannink et al., 2001). Loblolly pine is an ideal organism for association genetics because loblolly pine has natural and outcrossing populations distributed across large areas that have high gene-flow and little population substructure (Al-Rabab'ah and Williams, 2002; Brown et al., 2004b; Schmidtling et al., 1999). Also with loblolly pine it is possible to create large experimental populations and clonally propagate them to

detect, verify and evaluate phenotypes and genotypes. Loblolly pine also has desirable levels of nucleotide diversity (Θ=0.005; Brown et al., 2004) and limited linkage disequilibrium (<2500 bp on average, Brown et al., 2004).

As a first step toward dissecting complex disease traits in loblolly pine, I have undertaken this study to evaluate a variety of disease phenotypes in a population of 32 unrelated parents mated to form approximately 63 full-sib families that were clonally propagated to form hundreds or thousands of clones depending upon the experiment. Although an ideal association population would contain hundreds of unrelated individuals (McLeod and Long, 1999), this population is an excellent starting point to evaluate the heritabilities and genetic relationships among the two sets of disease traits that can then be dissected by association or QTL mapping.

An important aspect of experimental material is its clonal propagation to form a hierarchy of genetic relationships (parent, full-sib family, and clone) that facilitate the dissection of genetic architecture of diseases and their genetic relations. Since micro-environmental variation for a given genotype can be calculated using its vegetative propagules, I can obtain a more precise estimate of genetic components of disease resistance. Given that I am using the same genotypes to predict breeding values for both pitch canker and fusiform rust disease resistance, the values can be compared to look for correlations that will be informative when I try to understand the underlying genetic architecture.

The clonally propagated material of clones screened in the greenhouse conditions was also planted in several field sites. The first year fusiform rust disease incidence data from the naturally inoculated field site can be compared with the greenhouse screen to

confirm resistant and susceptible genotypes, since the ultimate goal of resistance screening is to identify the resistant genotypes that will be disease free in the plantations.

Genomic mapping has identified the region containing *Fr1* (fusiform resistance-1) conferring pathotype-specific resistance to fusiform rust (Wilcox et al., 1996). RAPD marker J7_485A was linked to the *Fr1* locus in progeny of a single loblolly pine parent. Thus, the progeny that have this marker were resistant whereas the ones without the marker were susceptible to fusiform rust incited by *C. quercuum* with the avirulence gene (Avr1). This genetic marker was consistently predictive of fusiform rust resistant trees in greenhouse (Kubisiak et al., 2005; Kuhlman et al., 1997) and field screens (Wilcox et al., 1996). Two families among 63 families that were screened for fusiform rust resistance in the greenhouse and the field were genotyped for *Fr1*. Thus, clones belonging to the two genotyped families can be used to verify the resistance prediction power of the genetic marker.

The genotypic information on progeny of the families that are segregating for the RAPD marker J7_485A can also be useful in molecular genetics studies. Microarray technology which became available with the last decade (ref) can be used to identify genes regulated in response to inoculation with *C. quercuum*. With the genetic marker information genetically resistant and susceptible individuals can be isolated to be challenged by *Fr1* avirulent strains of *C. quercuum*. The host responses, disease development and the interactions between the host and the pathogen can be revealed at the molecular genetics level.

CHAPTER 2
GENETIC DISSECTION OF FUSIFORM RUST AND PITCH CANKER DISEASE TRAITS IN LOBLOLLY PINE

Introduction

Pinus species are both economically and ecologically important. Pines grown in the southeastern United States generate nearly half of the nation's pulpwood, with an annual harvest value of approximately $19 billion (McKeever and Howard, 1996). Loblolly pine (*Pinus taeda* L.) is the most widely planted Pinus species in this region, averaging 74% of the annual seedling production (Carey and Kelley, 1993). In addition to plantations, loblolly pine is the predominant species on 11.7 million ha of native forest (Baker and Langdon, 1990), where it impacts the welfare of nearly 400 species of vertebrates (Schultz, 1999).

Loblolly pine is a host for two endemic pathogens, *Cronartium quercuum* Berk. Miyable ex Shirai f. sp. fusiforme (Burdsall and Snow, 1977), the inciting agent of fusiform rust disease, and *Fusarium circinatum* Nirenberg et O'Donnell (Nirenberg and O'Donnell, 1998), the inciting agent of pitch canker disease. Fusiform rust is one of the most destructive fungal diseases in the southeastern United States, causing damage ranging from $25–$135 million per year (Cubbage et al., 2000). The major symptom of fusiform rust disease is the formation of stem galls that lead to decreases in survival, wood quality, and growth. Genetic variation in resistance at the family level has been demonstrated for fusiform rust (Kuhlman and Powers, 1988; McKeand et al., 1999). Based on controlled inoculation studies carried out on specific loblolly and slash pine

Pinus elliottii Engelm. var. elliottii) families, specific resistance—i.e., "gene-for-gene" interactions—has evolved (Powers, 1980; Stelzer et al., 1997; Wilcox et al., 1996), as well as partial resistance in the form of short galls (Schmidt et al., 2000), which may be genetically distinct from specific resistance.

Pitch canker is also a significant, although more episodic, disease problem (Dwinell et al., 1985). Symptoms of pitch canker disease include resinous lesions on stems and branches that cause seedling mortality, decreased growth rates, and crown dieback (Dwinell et al., 1985). A considerable amount of genetic variation for pitch canker resistance has been detected in loblolly pine families (Kuhlman et al., 1982) and clones (Dwinell and Barrows-Broaddus, 1981); however, the genetic architecture of resistance is not well understood.

Our goal in this work was to obtain precise estimates of pitch canker and fusiform rust disease phenotypes expressed in loblolly pine. Precision was acquired by a combination of clonal propagation, which allows repeat observations of the same genotypes, and is now feasible in loblolly pine (Frampton et al., 2000), testing of over one thousand pedigreed genotypes, and the use of a mixed linear model (GAREML) to adjust for environmental effects (Huber, 1993). In this study, I identified traits, clones, families, and parents that guide a genetic approach to dissecting disease traits in loblolly pine. I verified that pitch canker and fusiform rust traits are heritable and identified the disease traits that are genetically distinct from one another. This work creates the baseline knowledge required for identifying genes that condition phenotypes of interest, either through genetic linkage analysis within defined pedigrees, or by association in populations of unrelated genotypes (Flint-Garcia et al., 2003; Jannink et al., 2001).

Materials and Methods

Genetic Material, Plant Propagation, and Experimental Design

The 63 loblolly pine families screened in this study were obtained from a circular mating design with some off-diagonal crossing. Members of the Cooperative Forest Genetics Research Program at the University of Florida and the North Carolina State University–Industry Cooperative Tree Improvement Program (Figure 2-1) provided the 32 parents and generated the full-sib families and clones screened in this study. Forty-nine seeds from each full-sib family were germinated and grown into hedges for clonal propagation. Maintenance of hedges and propagation of cuttings is reported in Baltunis et al. (2005). In brief, cuttings were set in July 2001, assessed for rooting after 9 weeks, and clones with the highest rooting ability selected for this experiment. The number of clones within families and the number of ramets (i.e., rooted cuttings) for each clone was not equal, since families did not produce the same number of clones, and clones had different rates of rooting. Cuttings assigned to a greenhouse screen were chosen at random from the ramet pool of each available clone (Table 2-1). The screens were grouped according to the disease (fusiform rust or pitch canker). The fusiform rust screens were conducted using two types of inoculum (a one-gall mix or a ten-gall mix), whereas both pitch canker screens used a single inoculum. The experimental design was a randomized complete block with single-tree plots arranged in an alpha lattice with an incomplete block size of 20. The clones were replicated with a maximum number of five ramets per experiment. Ramets were pruned twice to stimulate synchronous elongation of multiple succulent

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1		16	17				18										19															
2	**6**		18	17									20														18	19		22		
3	**34**	**23**		19	19																											
4		**69**	**37**			15																										
5			**58**			19	18				20		21																			
6				**15**	**10**		19	19							18																	
7	**45**				**11**	**64**		21	19																							
8						**50**	**56**		18	19													21									
9							**40**	**26**		20																						
10								**25**	**49**		21																					
11					**39**					**44**		18						21														
12											**28**		21	19											21							
13		**35**			**41**							**19**		19	21																	
14												**46**	**65**			18																
15						**7**							**68**			20	22															
16														**70**	**38**		22	21														
17	**16**														**36**	**42**		30	31													
18											**27**					**60**	**0**		21	22												
19																	**1**	**22**		22	15											
20																		**30**	**57**		21	20										21
21																			**13**	**29**			19									
22																				**8**			19	18					20			
23								**48**													**67**	**20**		18	20							
24																						**51**	**55**		17							
25												**66**											**43**	**62**		21	19					
26																									**9**		18	19				
27		**54**																							**31**	**32**		20				
28		**33**																								**52**	**21**		14	22		
29																						**3**					**2**					
30		**12**																									**53**					
31																																19
32																				**61**										**4**		

Figure 2-1 A circular mating design was used to generate the plant material. Thirty-two parents were crossed following a circular design, and the resulting progeny was used as the material screened for this study. The numbers in the cells above the diagonal are the number of clones used from a given cross, and the numbers below the diagonal are the family identification numbers

shoots for inoculation. The initial pruning occurred in March 2002, 8 months after setting, by cutting back the shoots from 10–15 cm to 3–4 cm each. The second pruning occurred 6 weeks prior to inoculations for both pitch canker and fusiform rust screens; shoots were succulent and 5–8 cm in length at the time of inoculation. After pruning, all trees were fertilized weekly with Miracle-Gro 15-30-15 until inoculation.

Pitch Canker: Inoculations and Data Collection

The larger of the two pitch canker screens was conducted at the USDA Forest Service Resistance Screening Center in Bent Creek, North Carolina, and is referred to as

Table 2-1 Summary of the four inoculation experiments reported in this study

Testa	# Families	# Clones	Rangeb	# Ramets	# Observationsc
RSC pitch canker	63	1065	7- 31	4483	7664
UF pitch canker	60	362	1- 24	1316	3119
Ten Gall fusiform rust	63	1360	17-31	5473	11,395
One Gall fusiform rust	63	698	2- 30	2743	5195

aRSC USDA Forest Service Resistance Screening Center, UF University of Florida
bNumber of clones within families
cNumber of observations exceeds the number of ramets because multiple shoots were assessed on a given ramet

the "RSC" screen in this manuscript. New growth (5–7 cm) was inoculated following the standard RSC protocol (Oak et al., 1987) with *F. circinatum* isolate S45 (Forest Pathology laboratory collection, University of Florida) at a density of 92,500 spores/ml. In brief, prior to spray inoculation, shoot tips were excised from two shoots on each ramet. After inoculation, plants were placed in a high-humidity chamber for 24 h, then transferred to a greenhouse and maintained at an average temperature of 20°C for 3 months.

The smaller of the two pitch canker screens was conducted at the University of Florida and is referred to here as the "UF" screen. Plants were pruned 6 weeks before inoculation with the same S45 isolate. One shoot tip per ramet was excised, and 1 µl of a 500-spores/µl solution was applied to the wound with a micropipette. All plants were incubated under high humidity for 24 h. The test was kept in the greenhouse for 36 days at an average temperature of 30°C.

Disease symptoms were measured at 90 days (RSC) and 36 days (UF). Shoot length and lesion length were measured (in millimeters) on one shoot chosen at random from each ramet at the RSC and on the single shoot inoculated per ramet at UF.

Both the RSC and UF pitch canker raw data sets included only one lesion-length and shoot-length measurement for each ramet. Prior to analysis, the data were standardized by experiment, using the phenotypic standard deviation calculated from the appropriate linear model for the screen.

Fusiform Rust: Inoculations and Data Collection

Plants were pruned twice before inoculation to stimulate elongation of multiple shoots per ramet. Both rust screens were inoculated at the RSC, following standard protocols (Knighten, 1988). The ten-gall test was inoculated at a density of 52,000 spores/ml with aeciospores pooled from a ten-gall collection obtained from a 6-year-old loblolly pine plantation near Lee, Florida (designated L-10-1-99, provided by Dr. Henry Amerson, NC State University) The one-gall test was inoculated at a density of 50,700 spores/ml with aeciospores isolated from a single gall obtained from a branch of slash pine family 84-57 near Callahan, Florida (designated #501, provided by Dr. Robert Schmidt, University of Florida).

Data were collected from both rust screens 6 months after inoculation. For each ramet with multiple shoots, the number of shoots with galls and the number of galls were counted and recorded. In addition, two shoots with single galls were randomly chosen to measure stem length, gall length, and gall width (in millimeters) for each ramet.

Data collected from both the ten-gall and one-gall screens were treated identically for gall measurements. Gall measurement values were averaged by ramet if there was more than one shoot with a single gall. Gall volume was calculated from gall length and gall width data, assuming a fusiform shape:

$$Volume = (3/4)length \times (width)^2$$

Ramets were scored as 0 (no gall) or 1 (at least one gall) for gall score. Ramets that did not form galls were not included in the gall length, width, and volume data. Gall length, width, and volume data sets were standardized using their respective phenotypic standard deviations calculated from the linear model.

Estimation of Genetic Parameters

Variance components and genetic parameters were estimated by GAREML (Huber, 1993), which employs restricted maximum likelihood estimation (Patterson and Thompson, 1971) and best linear unbiased prediction [(BLUP) Henderson, 1973]. The same linear model was applied to the traits measured in all four disease screens, since the experimental designs were identical. The linear model was:

$$y_{ijklm} = \mu + R_i + t(r)_{ij} + gca_k + gca_l + sca_{kl} + c(family)_{klm} + r * f_{ikl} + e_{ijklm}$$

where:

y_{ijklm} is the m^{th} observation of the k^{lth} cross in the j^{th} tray of ith rep.

μ is the population mean.

R_i is the fixed resolvable replication, i=1–5.

$t(r)_{ij}$ is the random variable tray incomplete block ~NID(0,σ^2_t), j=1–21.

gca_k is the random variable female general combining ability (GCA) ~NID(0, σ^2_{gca}) k=1–32.

gca_l is the random variable male general combining ability ~NID(0, σ^2_{gca}) l=1–32.

sca_{kl} is the random variable specific combining ability (SCA) ~NID(0, σ^2_{sca}).

$c(family)_{klm}$ is the random variable clone within a family ~NID(0, $\sigma^2_{c(family)}$).

$r*f_{ikl}$ is the random variable replication by family interaction ~NID(0, σ^2_{r*f})).

e_{ijklm} is the random variable error within the experiment ~NID(0, σ^2_e).

The narrow- (h2) and broad-sense (H2) heritabilities were calculated according to Falconer and Mackay (1996):

$$h^2 = \frac{4\hat{\sigma}^2_{gca}}{\hat{\sigma}^2_P} = \frac{\hat{V}(A)}{\hat{V}(P)}$$

$$H^2 = \frac{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)}}{\hat{\sigma}^2_P} = \frac{(\hat{V}(A) + \hat{V}(D) + \hat{V}(I))}{\hat{V}(P)}$$

where:

$\hat{\sigma}^2{}_P$ is the phenotypic variance,

$\hat{V}(P)$ is the total phenotypic variance,

$\hat{V}(A)$ is the additive variance,

$\hat{V}(D)$ is the dominance variance,

$\hat{V}(I)$ is the epistasis variance.

To partition the broad sense heritability I calculated the ratio of dominance variance to total phenotypic variance ($\hat{h}_D{}^2$) and the ratio of epistatic variance to total phenotypic variance ($\hat{h}_I{}^2$), using the following formulas:

$$\hat{h}_D{}^2 = \frac{4\hat{\sigma}^2_{sca}}{\hat{\sigma}_P{}^2} = \frac{\hat{V}(D)}{\hat{V}(P)}$$

$$\hat{h}_I{}^2 = \frac{\hat{\sigma}^2_{c(f)} - (0.50\hat{\sigma}^2_{gca} + 0.75\hat{\sigma}^2_{sca})}{\hat{\sigma}_P{}^2} = \frac{\hat{V}(I)}{\hat{V}(P)}$$

The broad sense heritability of clonal means ($H_C{}^2$) and family means ($H_F{}^2$) were calculated using the formulae below:

$$\hat{H}^2_C = \frac{(2*\hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)}}{(2*\hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)} + (\hat{\sigma}^2_{r*f}/r) + (\hat{\sigma}^2_e/r)}$$

$$\hat{H}_F^2 = \frac{(2*\hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca}}{(2*\hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca} + (\hat{\sigma}^2_{C(f)} / c) + (\hat{\sigma}^2_{r*f} / r) + (\hat{\sigma}^2_e / r*c)}$$

where r is the harmonic mean of ramets per clone and c is the harmonic mean of clones per family.

Family deviations were predicted by summing the following BLUP estimates produced by GAREML:

Family deviation = Predicted female value (gca_k) + Predicted male value (gca_l) + Predicted specific combining ability (sca_{kl})

Genetic Correlations

The genetic correlation between gall score and gall length at the parental, family, and clonal levels, and the correlation among screens within and across diseases were calculated on combined data sets by adding experiment × GCA (σ^2_{ge}), experiment by family (σ^2_{se}) and experiment by clone(family) ($\sigma^2_{c(f)e}$) interaction factors to the linear model and using the Type B genetic correlation formula (r_B; Yamada, 1962):

$$(r_B)_{\text{PARENTAL}} = \frac{\hat{\sigma}^2_{gca}}{(\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{ge})}$$

$$(r_B)_{\text{FAMILY}} = \frac{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca}}{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + 2\hat{\sigma}^2_{ge} + \hat{\sigma}^2_{se}}$$

$$(r_B)_{C(F)} = \frac{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{C(F)}}{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)} + 2\hat{\sigma}^2_{ge} + \hat{\sigma}^2_{se} + \hat{\sigma}^2_{c(f)e}}$$

Efficiency of using multiple ramets per genotype was calculated according to Huber et al. (1992):

$$Efficiency = (1 - H^2) / r$$

where r is the number of ramets per clone.

Results

The mating design shown in Figure 1, coupled with clonal propagation, allowed predictions of clonal, family, and parental genotypic values as well as population-wise estimates of heritabilities and genetic correlations of disease traits for both pathosystems. A total of 27,373 phenotypic data points were collected for lesion length (pitch canker), gall score, gall length, and gall width (fusiform rust). I first present data on pitch canker phenotypes, followed by fusiform rust, and finally a comparison of pitch canker and fusiform rust resistance.

Pitch Canker Disease Resistance is Heritable

The pitch canker disease screens performed at UF and RSC resulted in 89% of the ramets (i.e., rooted cuttings) showing measurable disease symptoms in each screen. BLUP clonal values were predicted for each screen, and the resulting distributions are shown in Figure 2a. The consistency of the disease rates and the shapes of the distributions (i.e., skewed to the right) suggest that statistical comparisons between the RSC and UF screens are appropriate. The genetic correlation between the RSC and UF screens was 0.88 at the parental level, 0.76 at the family level, and 0.69 at the clonal level. A scatter plot based on family ranks is presented in Figure 2b and reflects the positive correlation between the two screens. Therefore, I conclude that parents, families, and clones performed consistently across screens. After combining the data from the two screens, the five most resistant and the five most susceptible full-sib families were identified based on predicted family values and standard deviations for lesion length; these are indicated in Figure 2- 2a by their ID number from Figure 2-1. The resistant tail contains families 50 and 48, which have parent 8 in common. The resistant tail also contains half-sib families 61 and 4, which share parent 32. Resistant family 44 is not

related to any of the other resistant families in the tail (Figure 2-1). The susceptible tail is composed of three half-sib families. Susceptible family 12 has parent 30 in common with family 53. Family 12 also shares parent 2 with susceptible family 69. Susceptible families

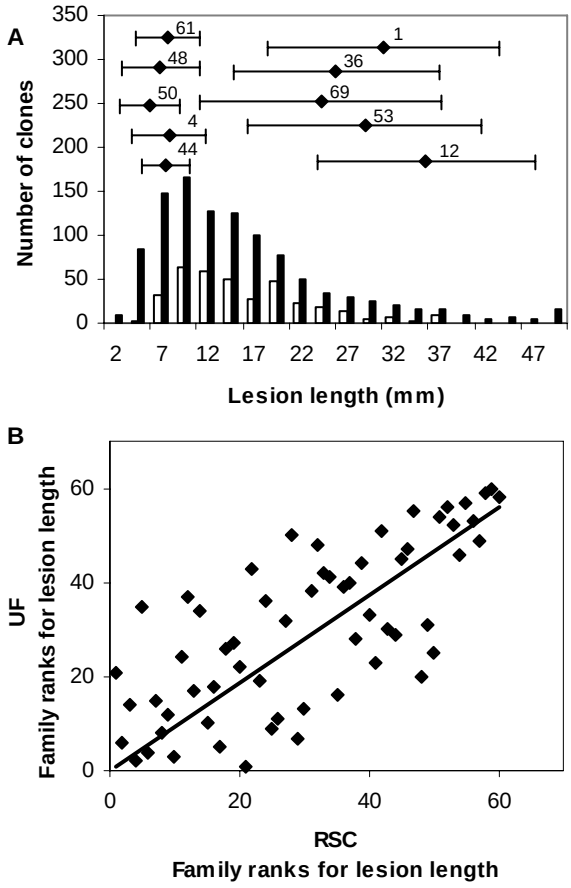

Figure 2-2 Frequency distributions and genetic correlation for pitch canker lesion length. (A) Distribution of best linear unbiased prediction (BLUP)-predicted clonal values for the USDA Forest Service Resistance Screening Center [(RSC) black] and University of Florida [(UF) white] pitch canker screens. Above the distribution are the predicted means and standard deviations of the five most susceptible and resistant families identified by their family ID number. (B) Ranks based on BLUP-predicted family values for RSC and UF were plotted against each other (a rank of 1 is the most resistant and 63 the most susceptible). A least squares regression line is shown after being forced through the origin due to a non-significant intercept.

1 and 36 have parent 17 in common. Families in the resistant tail and families in the susceptible tail did not have any parents in common, indicating no genetic relationships across the classes.

Two Distinct Inoculation Procedures Reveal Similar Heritabilities for Lesion Length

Using the RSC, UF, and pooled data, the heritabilities based on individual tree, family, and clonal means were calculated to determine how much of the variation in lesion length could be attributed to genetic variation and to determine the precision of the predicted clonal and family means. The broad-sense heritabilities for the clonal (H_C^2) and family (H_F^2) means were determined for both the individual and pooled pitch canker screens to evaluate the precision of the clonal and family means predicted above. HC2 and H_F^2 were greater for RSC (0.75) than for UF (0.61; Figure 2-3), because the number of ramets per clone and the number of clones per family were approximately three times greater for the RSC screen compared to the UF screen (Table 1). Narrow-sense heritabilities (h^2) for both the RSC and UF datasets were 0.27. Broad-sense heritabilities (H^2) were similar for both the RSC (0.43) and the UF screens (0.37) (Figure 2-3). When the RSC and UF data sets were pooled, heritabilities were not different from that calculated for each screen individually (Figure 2-3). This is another indicator that results from the two screens were comparable.

Disease Traits Associated with Fusiform Rust are Independently Inherited

The two fusiform rust screens are characterized by the type of inocula used, either ten-gall or one-gall. There were 36% and 31% galled ramets for the ten-gall and one-gall screens, respectively. A disease incidence (referred to as "score") dataset was generated

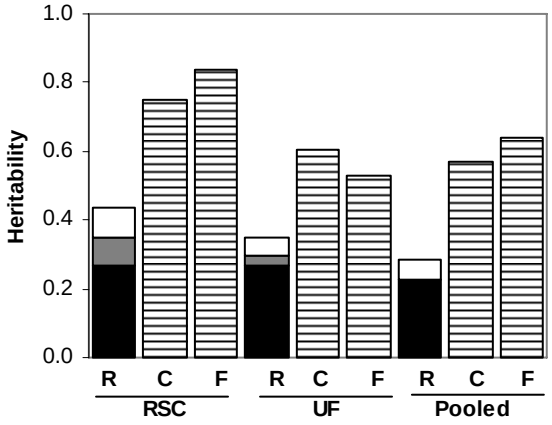

Figure 2-3 Heritability estimates for pitch canker lesion lengths. The bar graph shows the heritabilities for individual ramets ($R=H^2$) and the broad-sense heritabilities for clonal ($C=H_C^2$) and family ($F=H_F^2$) means for the RSC, UF, and pooled data. Narrow-sense heritability [(h^2) solid black], epistatic heritability [(h_I^2) solid gray], and dominance heritability [(h_D^2) white] are stacked so that the y-axis corresponding to the top of the bar is the broad-sense heritability.

by designating disease-free ramets as 0 and galled ramets as 1. Fusiform rust screens for score are shown in Figure 2-4a. The distributions for score in both screens follow a similar pattern, that is, there is a minor peak at a mean ~0.1, and the distribution is skewed to the right. In addition to disease incidence, gall length, and gall width were measured for ramets with galls. In contrast to score, the predicted clonal means for gall length revealed a normal distribution for both fusiform rust screens (Figure 2-4b). Because the distributions and overall disease incidences were similar, scaling prior to comparing the data from the two screens was not necessary for either trait.

Genetic correlations between the two screens were calculated for score and gall length in order to determine if inoculum type might impact trait expression. The genetic correlation for score was 0.80 at the parental level, 0.83 at the family level, and 0.86 at the clonal

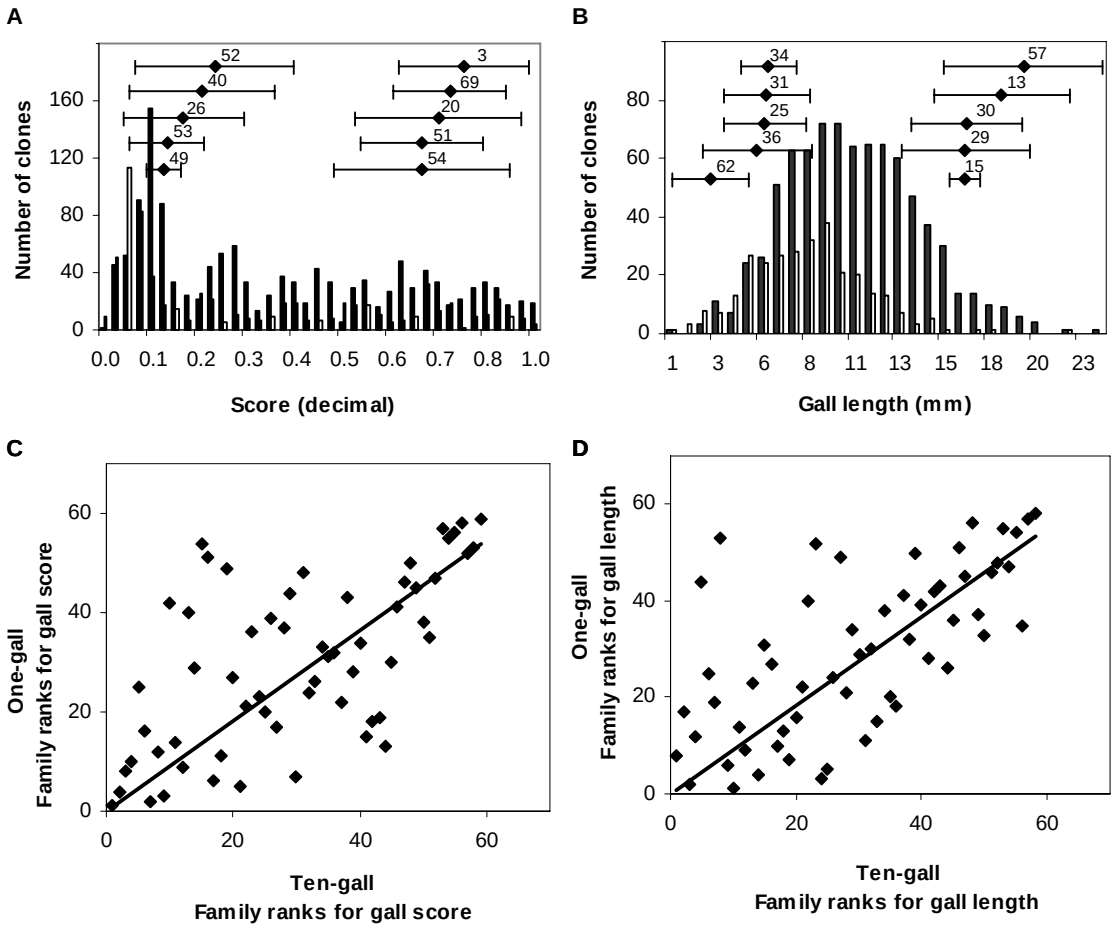

Figure 2-4 Frequency distributions and genetic correlations for fusiform rust disease traits. Distribution of BLUP-predicted clonal values for gall score (A) and gall length (B) in the ten-gall inoculum (black) and one-gall inoculum (white) screens are shown. Above the distribution are the predicted values and standard deviations of the five most susceptible and resistant families identified by their family ID number. Ranks based on predicted family values for gall score (C) and gall length (D) (1 = resistant, 63 = susceptible) are plotted against each other. A least squares regression line is shown after being forced through the origin due to a non-significant intercept

level, suggesting a general consistency in performance between the ten-gall and one-gall mixes. For gall length, the genetic correlation between the two screens was 1.00 at the parental level, 1.00 at the family level, and 0.76 at the clonal level, again indicating general consistency in performance between the two fusiform rust screens. Despite the high genetic correlations, I did observe "outlier" families that performed differently in

the two screens, suggesting some potentially significant genotype by inoculum interactions (Figure 2-4c, d).

Relationships among families with extreme phenotypes can reveal information regarding inheritance. For score, the predicted family values for the five most resistant and five most susceptible families are plotted on the graph in Figure 4a, along with their within-family standard deviations and family ID numbers. The resistant tail contains two half-sib family groups, that is, families 26, 40, and 49 that have parent 9 in common, and families 52 and 53 that have parent 28 in common. The susceptible tail is composed of two families that are half-sibs, that is, families 3, 20, and 51 have parent 22 in common, and families 54 and 69 have parent 2 in common. Similarly for gall length, the five families with the shortest galls and the five families with the longest galls are shown above the distribution in Figure 4b. The short gall-forming tail includes families 31 and 62 that have parent 25 in common. The remaining three families in this tail are unrelated. The five families with the longest galls comprise three half-sib families (13 and 29; 29 and 30; and 57, 13, and 57) that are related to one another through parents 21, 20, and 19, respectively. Family 15 is unrelated to the others. For both score and gall length, familial relationships within a given tail were common, whereas no such genetic relationships among families in opposing tails were observed. This is consistent with both score and gall length being heritable traits.

To evaluate how much of the trait variation associated with fusiform rust can be attributed to genetic effects, heritabilities were calculated. Since the genetic correlations for score and gall length were high across inocula (Figure 2-4c, d), data were pooled and used for heritability calculations. Gall score was consistently more heritable than gall

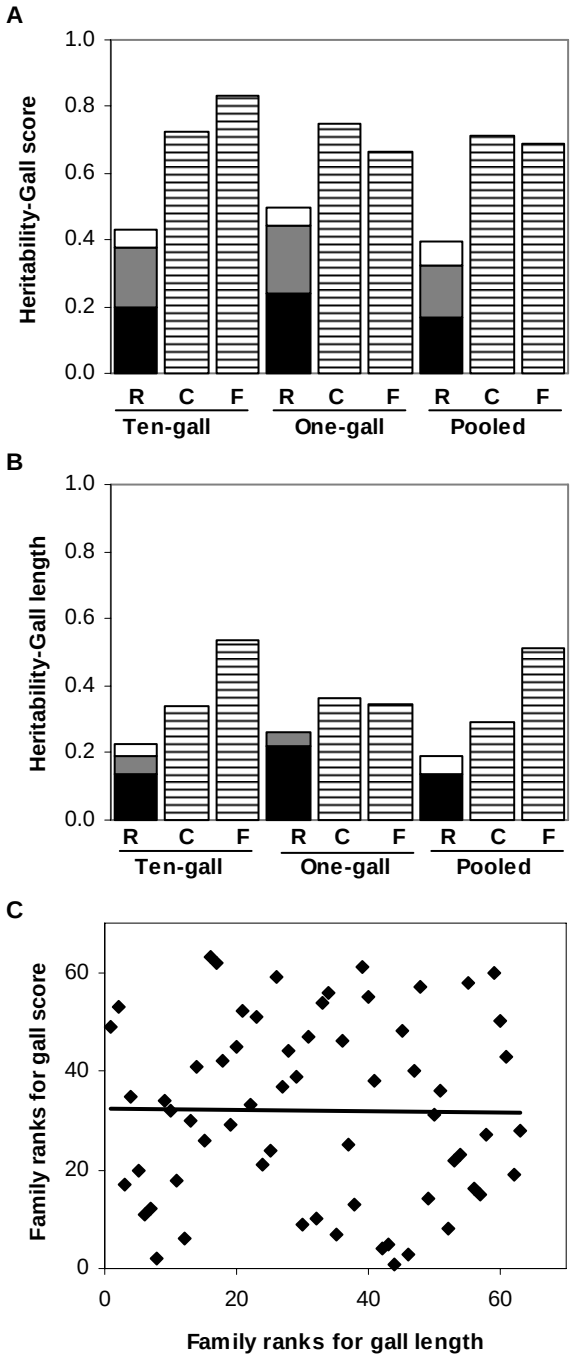

Figure 2-5 Heritability estimates and family rank scatter plots for fusiform rust disease traits. Gall score (A) and gall length (B) heritabilities for R, and C and F means are shown. Heritability estimates for the ten-gall and one-gall pooled screens are given for both traits. h^2 (solid black), h_I^2 (solid gray), and h_D^2 are stacked such that the y-axis corresponding to the top of the bar is the H^2. (C) Scatter plot of family ranks illustrates a lack of correlation between gall score and gall length traits (1 = resistant, 63 = susceptible)

used for heritability calculations. Gall score was consistently more heritable than gall length for the one-gall, ten-gall, and pooled datasets (Figure 2-5a, b).

Host Genes Underlying Resistance to Pitch Canker and Fusiform Rust are Independent

Necrotrophic (i.e., *F. circinatum*) and biotrophic (i.e., *C. quercuum*) pathogens have distinct life history properties. This implies that host genes underlying resistance may be different for diseases incited by necrotrophic and biotrophic pathogens. To determine whether host responses to *F. circinatum* and *C. quercuum* are independent, I computed the genetic correlations between lesion length (pitch canker) and the various gall characteristics (fusiform rust). There were no significant correlations between lesion length and gall length (Figure 2-6), or between lesion length and gall score (data not shown). The estimated genetic correlation between lesion length and gall length were 0.00 at the parental level, 0.00 at the family level, and 0.02 at the clonal level. No genetic correlations were found between lesion length and gall volume or gall width (0.00 for all, data not shown). Together, these results imply that resistance to pitch canker and resistance to fusiform rust are controlled by different host genes.

Efficiency of Using Multiple Ramets per Genotype

Theoretically, if the number of ramets per genotype is high enough, heritability estimates based on clonal means will be 1. To describe the relationship between the number of ramets and H_C^2 for disease traits investigated in this study, the efficiencies (Huber et al., 1992) for increasing number of ramets per genotype were plotted against the number of ramets (Figure 2-7), where efficiency is calculated as the average reduction

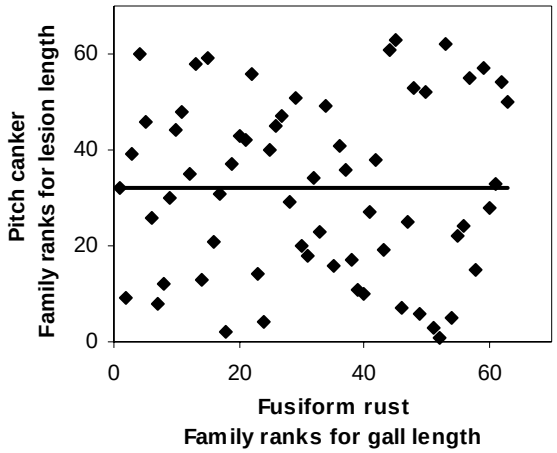

Figure 2-6 No genetic correlation between pitch canker and fusiform rust resistance. Family rank–rank scatter plot based on predicted family means for pitch canker (lesion length) and fusiform rust (gall length), fitted with a least squares regression line (1= resistant, 63= susceptible)

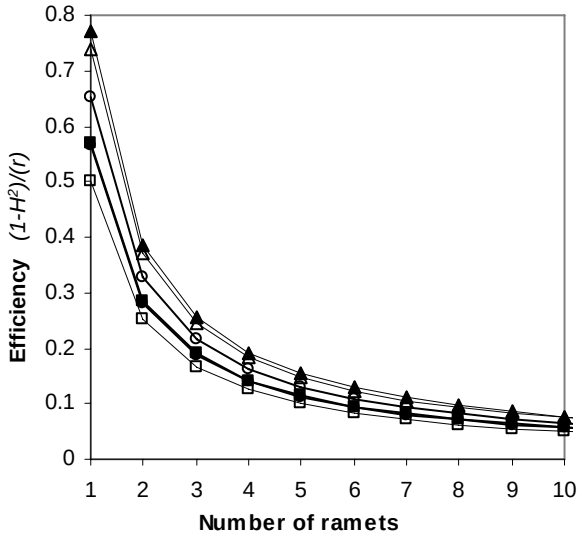

Figure 2-7 Efficiency is inversely proportional to the number of ramets per genotype. Efficiency of using multiple ramets in the estimation of HC2 plotted against number of ramets for RSC-lesion length (filled circles), UF-lesion length (open circles), ten-gall-gall score (filled squares), one-gall-gall score (open squares), ten-gall-gall length (filled triangles) and one-gall-gall length (open triangles). Efficiency was calculated as (1–H 2)/(number of ramets)

in error per ramet. For the different disease traits, the error associated with a clonal means decreases at different rates, depending on the number of ramets used to represent genotypes and H2. An increase in the experiment size above ca. five ramets per clone

does not appreciably increase the precision of heritability estimates, suggesting that future experiments of this type should be replicated to approximately the same extent as this study.

Discussion

Loblolly pine exhibits considerable variation in resistance to both fusiform rust (Kuhlman and Powers, 1988; Powers and Zobel, 1978) and pitch canker diseases (Dwinell and Barrows-Broaddus, 1981; Kuhlman and Cade, 1985). The pathogens that incite these diseases, the biotrophic fungus *C. quercuum* and the necrotrophic fungus *F. circinatum*, have distinct life history strategies, reflected in the contrasting disease symptoms visible on susceptible hosts. This study allowed a direct comparison of the host resistance mechanisms to these distinct pathogens in a common set of host genotypes. Consequently, it was possible to compare and contrast the genetic architecture of host responses to both pathogens.

Complex trait analysis requires a reliable estimation of phenotypic values for subsequent correlations with genotype. As a first step toward dissecting complex disease traits in loblolly pine, I undertook this study to evaluate a variety of disease phenotypes in a clonally propagated population generated via a circular mating design. Complex pedigree structures such as these can be useful for mapping QTL (Jannink et al., 2001).

Genetic variation for pitch canker resistance

Pitch canker resistance was continuously distributed across clones, suggesting that resistance may behave as a complex trait. Resistance to fungal necrotrophs is often inherited as a complex trait in crop species including maize (Bubeck et al., 1993) and rice (Wang et al., 1994). Another explanation for this continuous distribution is Mendelian inheritance of resistance within families that appears continuous when examined across

families. If resistance were monogenic, some families would be expected to show a bimodal distribution for lesion length. To assess this possibility, I tested individual families for bimodal distributions of resistance. None of the within-family distributions was bimodal; all showed continuous distributions. Since lesion length showed a continuous distribution within families across the entire study, I infer that pitch canker resistance is appropriate to analyze as a complex trait.

The repeatability of the pitch canker resistance screens was high, indicated by the high genetic correlation between the two screens, one of which was based on hand-inoculation in a warm environment (UF screen) and the other using established spray inoculation methods in a cooler environment (RSC screen). The stability of H^2 in the pooled dataset relative to the individual screens also supports this conclusion. I do not expect pathogenic variation to significantly change the resistance rankings of these genotypes, even though these experiments were performed by inoculating hosts with a single clonal isolate of *F. circinatum*. This is because there is little evidence for specific resistance in this pathosystem; families rank consistently when challenged with different fungal isolates (G. Blakeslee, personal communication). The facultative nature of this pathogen presumably creates little selection pressure for the evolution of gene-for-gene specificity in this pathosystem. Consequently, these clonal rankings may be robust across a broad range of pathogen isolates and predictive of rankings expected in the clonal field trials established with these genotypes.

While narrow-sense heritability is an important metric for breeding applications, our use of clonally replicated material allowed additional heritability calculations, H^2, H_C^2, and H_F^2 values, which take advantage of the mating and propagation designs used in

this study. H_C^2 is an appropriate metric for association and quantitative trait loci studies, because genotyping and phenotyping are both done at the clonal level. Accordingly, in the RSC screen (which involved the most genotypes of the two pitch canker screens) ca. 75% of the variation in lesion length at the clonal mean level was due to genetic variation. Therefore, I expect lesion length to be an appropriate phenotypic trait for future QTL identification.

Gall score and gall length are the most heritable fusiform rust traits

Our analysis of gall score (i.e., disease incidence) revealed a non-normal, right-skewed distribution with one major peak and several minor peaks. The major peak of apparently "resistant" genotypes may reflect an overestimation of host resistance because of the use of rooted cuttings. Studies comparing the responses of seedlings to rooted cuttings have revealed that these two types of plant material behave differently in response to pathogen challenge, with rooted cuttings showing enhanced resistance (Foster and Anderson, 1989; Frampton et al., 2000). This enhanced resistance phenomenon has been observed in other species and is often referred to as "age-dependent" resistance because the developmental stage of the infected organ is the key driver of resistance, over and above the action of specific resistance genes (Kus et al., 2002). As clonal host materials become more widely used in research and plantation forestry, our understanding of this phenomenon should improve.

Evidence for specific resistance in the loblolly pine–*C. quercuum* pathosystem has been obtained using genomic mapping (Wilcox et al., 1996) and by inference based on family rank changes in response to genetically distinct pathogen cultures (Kuhlman, 1992; Powers, 1980; Stelzer et al., 1997). Although the overall consistency among clonal performances in our two screens was high, I observed a few family and clonal rank

changes for particular families and genotypes between the ten-gall and one-gall inoculations (see outliers in Figure 2-4c), suggesting resistance genes in the host population interacted with specific pathotypes in the inocula. The families showing rank changes between the two inocula may provide a good starting point for identifying additional resistance genes in loblolly pine.

Gall length was normally distributed and was heritable, although to a lesser extent than gall score. Gall length could only be measured on a subset of the population (i.e., on galled ramets), and this sampling effect may account in part for the reduced heritability estimates. Our rationale for measuring gall size characteristics was based on work in slash pine (Schmidt et al., 2000) suggesting that families exhibiting small (short) gall phenotypes were expressing partial resistance to fusiform rust, based on their lack of subsequent sporulation. Partial resistance may be a more durable form of resistance given that it is often race nonspecific (Schmidt et al., 2000 and references therein). I observed continuous variation in gall length in loblolly pine and found no changes in the relative rankings of genotypes that formed galls in both screens as indicated by high genetic correlations (Figure 2-4d). Thus, inoculum type did not appear to exert a major effect on gall length. Studies involving a number of defined pathogen cultures will be required to resolve the question of whether gall length is conditioned by (relatively late-acting) specific resistance factors, or if gall length is a complex trait, potentially involving multiple genes with small effects.

The relationship between gall score and gall length was of interest, because these are distinct phenotypes whose genetic relationship is not well understood. The lack of genetic correlation between gall score and gall length, and the lack of relatedness among

families in the tail distributions for gall score and gall length both suggest that distinct gene systems condition these two traits. Previous studies have revealed that mean gall length varies substantially in loblolly pine families phenotyped as "resistant" based on score (Kuhlman, 1992), providing further support for the conclusion that gall score and gall length are conditioned by distinct genetic mechanisms. Future identification of QTL underlying gall length should help distinguish these loci from resistance genes known to be associated with gall score in loblolly pine (Wilcox et al., 1996).

Resistance to pitch canker and fusiform rust are under the control of two different mechanisms

The lack of genetic correlation between pitch canker resistance and fusiform rust resistance (as measured by gall score, or gall length) is consistent with distinct genetic architectures underlying host resistance to these two diseases. Biotrophic pathogens suppress host defenses because they require living host cells for survival and nutrient uptake. Hosts resistant to biotrophic pathogens often activate a localized cell death response to prevent spread of the pathogen (Thomma et al., 2001). In contrast, necrotrophic pathogens actively destroy host cells and utilize the released nutrients for survival. Therefore, a host-cell death-response effective against biotrophic pathogens is postulated to benefit necrotrophic pathogens by increasing nutrient availability through accelerated host tissue destruction. I propose that resistance to the necrotrophic pathogen *F. circinatum* is mechanistically distinct from resistance to the biotrophic pathogen *C. quercuum* due to the differing strategies employed by the two pathogens to incite disease in the host. This is supported by gene-expression array data, which revealed a lack of regulation of rust-associated genes after challenge by Fusarium (Morse et al., 2004). Although I identified families with excellent resistance to both diseases (Figure 2-6),

disease resistance to the two pathogens should be regarded as independent traits by breeders.

Phenotyping for disease trait dissection in loblolly pine

The work described in this manuscript has assigned specific phenotypic values to more than 1,000 loblolly pine genotypes, enabling the identification of genes and alleles that condition resistance through association studies. Genotyping and association studies are currently underway (ADEPT project Web site, Allele Discovery of Economically-important Pine Traits, http://dendrome.ucdavis.edu/ADEPT/) for candidate loci (Morse et al., 2004) thought to be involved in disease resistance in loblolly pine.

In this study, I increased the precision of phenotyping by using clonally propagated genotypes and mixed linear modeling to adjust for environmental effects. Increasing the number of ramets for a given clone will increase the clonal mean based heritability for use in linkage or association studies. However, there is a point of diminishing returns beyond which adding more ramets does not increase precision of phenotyping. This population was an excellent starting point to evaluate the heritabilities and relationships among disease traits. Furthermore, it should afford an opportunity to identify QTL by linkage and linkage disequilibrium (i.e., association) mapping, which has been proposed (Wu et al., 2002) and applied with success (Farnir et al., 2002; Meuwissen et al., 2002).

CHAPTER 3
FUSIFORM RUST RESISTANCE COSEGREGATES WITH AN *FR1*-LINKED MARKER AND REVEALS VARIABLE PENETRANCE OF THE DISEASE PHENOTYPE

Introduction

The economic value of pine in the southeastern United States exceeds $19 billion annually with this region supplying more than half of the nation's pulpwood (McKeever and Howard, 1996). Loblolly pine (*Pinus taeda* L.) is the primary pine species in the region, covering 45% of the commercial forest land (Schultz, 1999) with annual production of over 1 billion seedlings for planted in reforestation programs (McKeand et al., 2003).

Successful plantation establishment in the southeastern United States is highly dependent on the resistance of the planting stock to fusiform rust disease, which is incited by the endemic pathogen, *Cronartium quercuum* Berk. Miyable ex Shirai f. sp. fusiforme (Burdsall and Snow, 1977). The major symptom of fusiform rust disease is the formation of stem galls which decrease survival, wood quality, and growth, causing an annual loss ranging from $25–$135 million (Cubbage et al., 2000). Loblolly pine families exhibit substantial genetic variation in resistance to fusiform rust disease (Kuhlman and Powers, 1988; McKeand et al., 1999) both in greenhouse and the field.

Genomic mapping has identified the region containing *Fr1* (fusiform resistance-1) conferring pathotype-specific resistance to fusiform rust (Wilcox et al., 1996). RAPD marker J7_485A was linked to the *Fr1* locus in progeny of a single loblolly pine parent. Thus, the progeny that have this marker were resistant whereas the ones without the

marker were susceptible to fusiform rust incited by *C. quercuum* with the corresponding avirulence allele (Avr1). This genetic marker was consistently predictive of fusiform rust resistant trees in greenhouse (Kubisiak et al., 2005; Kuhlman et al., 1997), and field screens (Wilcox et al., 1996).

Screening of fusiform rust disease on clonally propagated loblolly pine has revealed the existence of "escapes"; ramets that are genetically susceptible yet do not show any disease symptoms (Foster and Anderson, 1989; Frampton et al., 2000). The basis for an "escape" can be a passive form of resistance; a random phenomenon where some cuttings harden off faster than others because of local environment within an experimental block. Alternatively, there may be a genetic basis for disease resistance, which may occur, for example if certain genotypes develop succulent shoots in response to hedging and fertilization at different rates or to different extents than other genotypes. A genetic analysis can answer this question.

Biologically a clone is susceptible if it has at least one diseased ramet. In this study I used the same approach which led us to use a genotype based analysis rather than a ramet based. I used some of the DNA markers developed in previous mapping studies to distinguish host genotypes that carry/lack the pathotype-specific *Fr1* allele. I tested the hypothesis that the *Fr1* allele is predictive of resistance in greenhouse and field experiments. Because these studies involved clonally propagated materials, I also quantified the extent to which genetic and non-genetic factors influence disease expression levels and escape rate in greenhouse and field trials.

Materials and Methods

Genetic Material

All the clones that were screened in the greenhouse and the field came from 63 full-sib loblolly pine families obtained from a circular mating design among 32 unrelated parents with some off-diagonal crossing. The genetic material, the propagation methods, the inoculations and the data collection were described in (Kayihan et al., 2005). The parents were from the Atlantic Coastal Plain and Florida provenances of loblolly pine. Briefly, there were 7-21 clones per full-sib family depending on the family and experiment and approximately 4 ramets per clone.

Genotyping Families 0 and 1 for Fr1

Among 32 parents used in this study, parent number 17 was recognized as heterozygous for pathotype-specific resistance gene *Fr1* (*Fr1*/*Fr1*; (Wilcox et al., 1996)). Full-sib families 0 and 1 were generated by crossing parent number 17 with parents 18 and 19 (Kayihan et al., 2005) which were known to be *Fr1*/*Fr1* (unpublished data) and a total of 61 clones from these families were genotyped using the protocols described in Wilcox et al. (1996). The J7_470 RAPD marker is linked to the *Fr1* locus and therefore could be used to predict seedling genotypes (*Fr1*/*Fr1* or *Fr1*/*Fr1*). The mating design (Kayihan et al., 2005) coupled with clonal propagation allowed direct assessment of marker-trait co-segregation. Because parents 18 and 19 are homozygous for the *Fr1* allele (recessive), families 0 and 1 are test-cross progeny and segregate 1:1 for resistance to *Fr1* avirulent (Avr-*Fr1*) inoculum. Since the maternal parent is heterozygous, megagametophyte samples were genotyped at the onset of the study to predict seedling genotypes. At the conclusion of the greenhouse screen, foliage samples were collected

from galled ramets that had been initially genotyped as *Fr1*/*Fr1*, and the genotyping reactions were repeated on the foliar DNA.

Greenhouse screen

The experimental design was a randomized complete block with single-tree plots arranged in an alpha lattice with an incomplete block size of twenty. Propagation of cuttings was described in Baltunis et al.(2005). A total of 63 families were used to generate 1360 clones for the ten-gall screen and 699 clones for the one-gall screen (Table 3-1). The clones were replicated with a maximum number of five ramets per experiment (Kayihan et al., 2005).

Table 3-1 Summary of the greenhouse and field screens reported in this study. The 63 families and most of the clones screened were the same across the ten gall, one gall and field screens. Percentage of diseased ramets and clones are reported as a measure of infection rate.

Screen	# of families	# of clones	# of ramets	% of clones galled	% of ramets galled
Ten gall	**63**	**1360**	**5473**	**62**	**36**
One Gall	**63**	**698**	**2743**	**49**	**31**
Field	**60**	**868**	**3362**	**51**	**26**

The ten-gall test was inoculated with aeciospores pooled from a ten-gall collection from Madison, FL (designated L-10-1-99) (Figure 3-1). The ten-gall inoculum was tested for virulence against *Fr1*; I inoculated 100 open-pollinated seedlings derived from parent 17, using 50,000 basidiospores/ml and RSC standard methods (Knighten, 1988). Ninety-four out of ninety-six seedlings that were chosen for DNA analysis were scorable for the RAPD marker J7_470 and the marker data obtained from the megagametophytes of these seedlings were used to detect virulence against *Fr1*. In order to choose an inoculum with the least amount of genetic diversity for the one-gall screen, aeciospores collected from single galls on slash pines in a field site (a generous gift from Dr. Robert Schmidt) were assessed by Simple Sequence Repeat (SSR) markers using the methods described by

(Kubisiak et al., 2004). The analysis showed that all four of the single gall samples contained at least four or more SSR haplotypes, indicating a minimum of four fungal pathotypes in each gall (data not shown). The single gall spore collection (designated #501) from Nassau, FL (Figure 3-1) was chosen from for inoculation because of its low genetic diversity, however its virulence against *Fr1* was not known prior to this study. The artificial inoculation procedures are described in Kayihan et al. (2005) and ramets were assessed for the presence (1) and absence (0) of a gall 6 months after the inoculation.

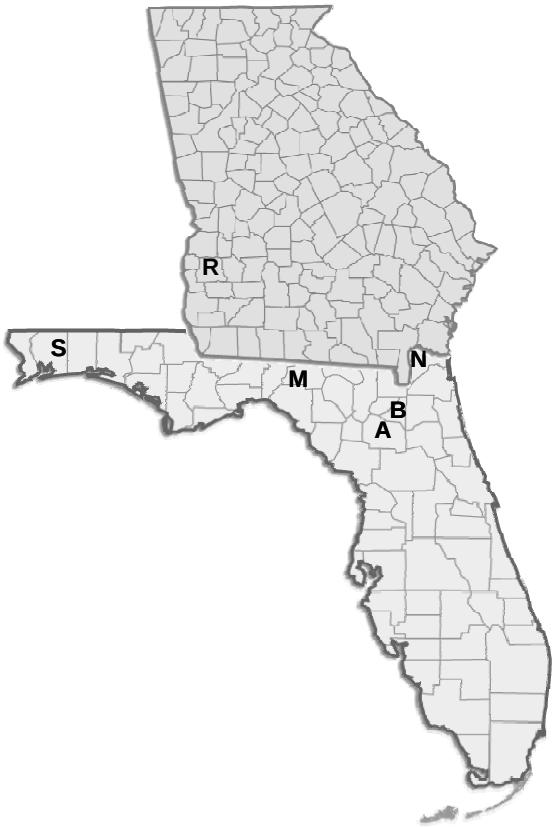

Figure 3-1 The inoculum sources used in the ten-gall (Madison County, FL) and one-gall (Nassau County, FL) greenhouse trials and field screens (Randolph County, GA) mapped in Florida and Georgia along with the other areas that were assessed for virulence. Virulence against *Fr1* was not detected in the inoculum obtained from these counties (S=Santa Rosa, A=Alachua, B=Bradford, M=Madison, R=Randolph, N=Nassau; personal communication Dr. Henry Amerson).

Field

The experimental design was a randomized complete block, with single-tree plots arranged in an alpha lattice with row/column family restrictions and an incomplete block size of five. A total of 868 clones from 60 families shared with the greenhouse screens were evaluated for fusiform rust disease resistance in the field (Table 3-1). The field location was Randolph, GA at latitude 31.78N, longitude 84.8W (Figure 3-1). Trees were planted in 4 replications each with 40 columns and 110 rows, inoculation was allowed to occur naturally and the cuttings were phenotyped for presence (1) or absence (0) of fusiform rust galls during the second growing season. This is an area classified as "high hazard" for fusiform rust disease based on south wide classification of >30% of stems in 5 to 15 years old stands that are likely to have galls on main stems or on live limbs that are fairly close to the main stem (Anderson et al., 1988).

Data Analysis

Conceptually, all the ramets from a resistant clone should be disease free and all ramets from a susceptible clone should be diseased. In this study, a clone was labeled as "resistant" when all of the ramets from that particular clone were disease free. However, there were a considerable number of cases where only some of the ramets from a susceptible clone were galled. For the purposes of this study, a single ramet bearing one or more galls was sufficient to identify a susceptible clone. I converted "ramet based score" data to a "clone based" dataset by classifying all the genotypes (i.e. clones) with one or more galls as susceptible (1) and the ones with no galls at all resistant (0).

I used families 0 and 1 as a measure of resistance, since both of these families included clones that were *Fr1*/*Fr1* or *Fr1*/*Fr1*. A few cases of no marker-trait co-segregation could be explained by genetic recombination between the marker and *Fr1*.

To evaluate the probability of a crossover between the molecular marker J7_485A and the *Fr1* locus in a given family I used the formula;

$$f(x) = \binom{n}{x} p^x (1-p)^{n-x}$$

where

n is the number of clones in the given family,

x is the number of clones that are putative recombinants in the given family,

p is the recombination fraction.

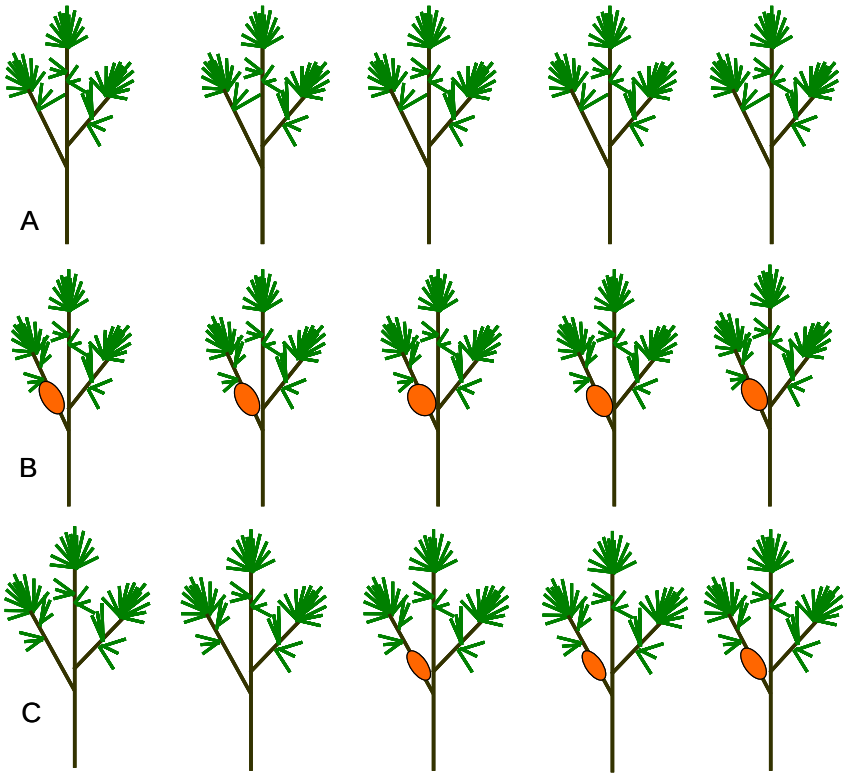

Figure 3-2 Diagrams illustrating genotype (clone) based phenotyping for disease resistance, susceptibility and escape rate. (A)A clone was declared resistant with five ramets were disease free. Resistant genotypes were not included in calculations of 'escape rate'. (B) An illustration of a "susceptible" clone with five galled ramets (i.e., an escape rate of 0%). (C)An illustration of a susceptible clone with three galled and two disease free ramets (i.e., escape rate of 40%).

I defined "escape rate" as the ratio of ramets that did not exhibit a gall, to the total number of ramets, given a clone that had at least one galled ramet (Figure 3-2).

$$E_r = \frac{r_t - r_g}{r_t}$$

where,

E_r is escape rate

r_t is total number of ramets for a given clone, and

r_g is the number of ramets with galls for a given clone

When all the ramets from a clone are disease free, without genetic marker information one can not distinguish a true genotype-level escape from a clone harboring additional resistance determinants. Therefore, genotypes that lacked diseased ramet were deleted from this dataset. Since the escape rate dataset was formed using the percentage of disease-free ramets in otherwise susceptible clones, this dataset was "clone based".

The "clone based" datasets from all 63 families from the two greenhouse screens (ten-gall and one-gall) and field screen were analyzed to understand genetic control of both score (susceptible or resistant) and escape rate. Variance components and genetic parameters were estimated by GAREML (Huber, 1993) which employs restricted maximum likelihood estimation (REML) (Patterson and Thompson, 1971) and best linear unbiased prediction (BLUP) (Henderson, 1973). This approach also aided a more valid comparison of the score and the escape rate datasets. The linear model used to analyze the 'escape rate' and the score dataset was:

$$y_{klm} = \mu + gca_k + gca_l + sca_{kl} + e_{klm}$$

where,

y_{klm} is the mth clone of the k^{lth} full-sib family,

μ is the population mean,

gca_k is the random variable female general combining ability (GCA) ~NID(0,σ^2_{gca}) k=1 to 32,

gca_l is the random variable male general combining ability ~NID(0,σ^2_{gca}) l=1 to 32,-

sca_{kl} is the random variable specific combining ability (SCA) ~NID(0,σ^2_{sca}),

e_{klm} is the random variable error within the experiment ~NID(0,σ^2_e).

The narrow (h^2) and broad (H^2) sense heritabilities were calculated according to (Falconer and Mackay, 1996) based on 0, 1 data for disease incidence (gall score) and decimal equivalent for escape:

$$h^2 = \frac{4\hat{\sigma}^2_{gca}}{\hat{\sigma}^2_P} = \frac{\hat{V}(A)}{\hat{V}(P)}$$

$$H^2 = \frac{4\hat{\sigma}^2_{gca} + 4\hat{\sigma}^2_{sca}}{\hat{\sigma}^2_P} = \frac{(\hat{V}(A) + \hat{V}(D))}{\hat{V}(P)}$$

where:

$\hat{\sigma}^2_P$ is the phenotypic variance,

$\hat{V}(P)$ is the total phenotypic variance,

$\hat{V}(A)$ is the additive variance,

$\hat{V}(D)$ is the dominance variance.

Genetic Correlations

The genetic correlation at the family level between the ten-gall and the field screens was calculated on combined data sets by adding Experiment by GCA (σ^2_{ge}) and

Experiment by Family (σ^2_{se})interaction factors to the linear model and using the Type B genetic correlation formula (rB; Yamada 1962):

$$(r_B)_{FAMILY} = \frac{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca}}{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + 2\hat{\sigma}^2_{ge} + \hat{\sigma}^2_{se}}$$

Asymptotic Z-test

In the field test, there was a possibility of uneven inoculation a result of natural dispersion of inocula. With the purpose of investigating this possibility I run the following model in ASREML(Gilmour et al., 2004);

$$y_{klm} = \mu + r_i + c_j + w_k + (r*c)_{ij} + (r*w)_{ik} + gca_l + gca_m + sca_{lm} + clone(family)_{lmn} + (r*sca)_{ilm} + e_{ijklmn}$$

μ is the population mean,

r_i is the random replication ~NID(0,σ^2_r), i=1 to 4,

c_j is the random variable column incomplete block ~NID(0,σ^2_c), j=1 to 40,

w_k is the random variable row incomplete block ~NID(0,σ^2_w), j=1 to 110,

gca_l is the random variable female general combining ability (GCA) ~NID(0,σ^2_{gca}) k=1 to 32,

gca_m is the random variable male general combining ability ~NID(0,σ^2_{gca}) l=1 to 32,

sca_{lm} is the random variable specific combining ability (SCA) ~NID(0,σ^2_{sca}),

$clone(family)_{lmn}$ is the random variable clone within a family ~NID(0,$\sigma^2_{c(family)}$),

$(r*sca)_{ilm}$ is the random variable replication by family interaction ~NID(0,σ^2_{r*sca})),

e_{ijklmn} is the random variable error within the experiment ~NID(0,σ^2_e).

Asymptotic Z-test for r_i, c_j, $(r*c)_{ij}$, $(r*w)_{ik}$ and $(r*sca)_{ilm}$ were calculated by dividing the variance of these components by corresponding standard deviation.

Results

Inheritance of Fusiform Rust Resistance in the Greenhouse and Field

In a previous report (Kayihan et al., 2005) I calculated fusiform rust disease resistance for genotypes and disease incidence (score) based on the proportion of ramets that were diseased for each clone. In this study a clone with at least one diseased ramet was classified as susceptible (1) and a clone with no diseased ramet was classified as resistant (0). Clone based resistance to fusiform rust disease (score) was an equally heritable trait both in the greenhouse and the field screens. Narrow sense heritabilities (h^2) for gall score were the highest in one-gall screen and the lowest in the field screen (Table 3-2). The broad sense heritabilities (H^2) for both the greenhouse and the field screen were high for this trait (Table 3-2). H^2 was highest in one-gall screen which was followed by ten-gall screen. The field screen yielded the lowest H^2 among these screens. Comparison of h^2 with H^2 within each screen showed that general combining ability (GCA) was higher then specific combining ability (SCA) in all trials.

Table 3-2 Summary of score (disease incidence) and escape rate datasets along with narrow sense heritabilities (h2) and broad sense heritability (H2) for escape rate and score in ten-gall, one-gall and field fusiform rust screens. All the families analyzed in one-gall and field were a subset of the families screened in ten-gall.

	Score dataset				**Escape dataset**			
	# of families	**# of clones**	**h^2**	**H^2**	**# of families**	**# of clones**	**h^2**	**H^2**
Ten gall	**63**	**1360**	**0.39**	**0.46**	**62**	**443**	**0.23**	**0.29**
One gall	**63**	**699**	**0.43**	**0.52**	**59**	**337**	**0.29**	**0.30**
Field	**60**	**868**	**0.31**	**0.36**	**61**	**439**	**0**	**0**

The ten-gall and one-gall greenhouse screens were highly correlated (Kayihan et al, 2005). According to the genetic correlations I calculated between the ten-gall and the

field screen for score (disease incidence), the families performed consistently, yielding high genetic correlation (r_f = 0.83) (Figure 3-3).

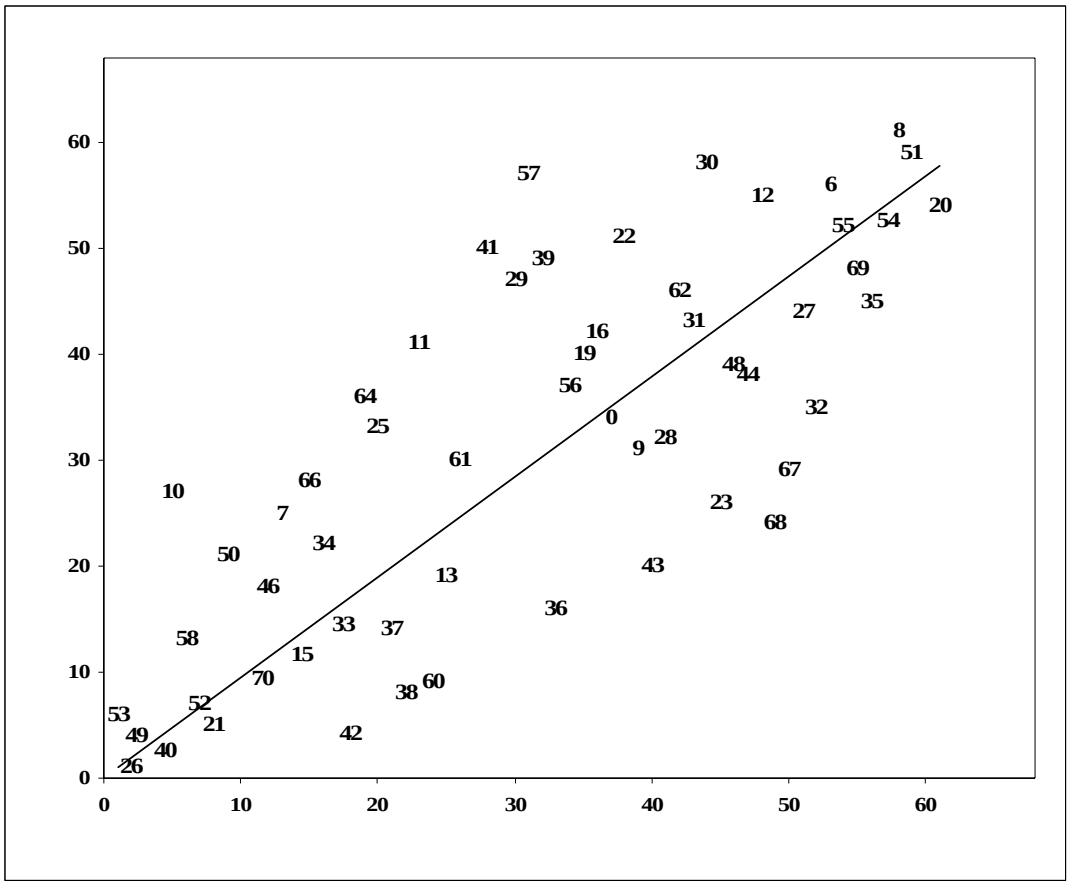

Figure 3-3 Scatter plot of ranks based on BLUP-predicted family genetic values for ten-gall and field were plotted against each other (a rank of '1' is the most resistant and '63' the most susceptible). A least squares regression line is shown after being forced through the origin due to a not-significant intercept. The numbers shown are the family identification codes for the full-sib families.

Validation of Fr1 marker

Prior to the greenhouse screen using the rooted cuttings, the ten-gall inoculum was tested for virulence against *Fr1* with use of *Fr1*/*Fr1* parent 17 seedling progeny in North Carolina State University (personal communication Dr Henry Amerson). From the 94 seedlings that were inoculated with ten-gall inoculum, 47 were genotyped *Fr1* (resistant)

and 47 were *Fr1* (susceptible). Of the 47 resistant seedlings, none were galled 9 months after inoculation and in the susceptible group 34 of 47 trees were galled. Hence infection in the *Fr1* group was 72%, while infection in the *Fr1* group was 0%. There was no evidence of virulence against *Fr1*. Also infection in Resistance Screening Center (RSC), Asheville, NC standard susceptible check for loblolly pine (10-8-3) was 76%, so the susceptible check lot and the *Fr1* group had the same amount of infection.

Fr1 marker data can be used to make inferences about resistance to fusiform rust disease in families from parent 17. I had three screens (ten-gall, one-gall and field) to contrast and compare the relative resistance levels of two genotyped families 0 and 1. Similar percentages of disease incidence in ten-gall, one-gall and field screens at both the ramet (36%, 31% and 26% respectively) and the clone (62%, 49% and 51% respectively) levels, gave us confidence to compare them without further adjustment (Table 3-1).

All megagametophytes from families 0 and 1 were genotyped and classified as either *Fr1* or *Fr1*. The marker J7_485A cosegregated with presence/absence of galls in families 0 and 1 in the greenhouse and field screens (Table 3-3). In families 0 and 1 most of clones behaved as expected. In the ten-gall screen, twenty four out of twenty nine clones that were *Fr1*/*Fr1* were disease free whereas twenty six clones were disease free out of thirty clones that were *Fr1*/*Fr1*. In both the one-gall and the field screens, all the clones from family 0 and 1 that were *Fr1*/*Fr1* , did not have any disease symptoms as expected. Twenty two out of twenty five clones that were *Fr1*/*Fr1* were diseased in the one gall and twelve out of sixteen that were genetically susceptible were galled in the field test. As I stated before ten-gall inoculum collected from Madison County, FL was avirulent to *Fr1*.

Table 3-3 Segregation of marker J7_485A linked to *Fr1* gene in families 0 and 1 across ten-gall, one-gall and field screens (658 ramets combined) with disease phenotype. Parent number 17 is heterozygous for pathotype-specific resistance gene *Fr1*. Family 0 is full sib test crosses between parent 17 (*Fr1*/*Fr1*) and 18 (*Fr1*/*Fr1*), whereas family 1 is test cross between 17 (*Fr1*/*Fr1*) and 19 (*Fr1*/*Fr1*). Gray cells highlight the clones that were not in the expected class.

Inoculum	**Ten gall**				**One gall**				**Field**			
Genotype *Fr1* (+) or *fr1* (-)	**+**		**-**		**+**		**-**		**+**		**-**	
Gall	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
Family 0	**2**	**13**	**11**	**3**	**0**	**14**	**10**	**0**	**0**	**9**	**7**	**1**
Family 1	**2**	**13**	**13**	**2**	**0**	**15**	**12**	**3**	**0**	**9**	**5**	**3**
Overall observed	**4**	**26**	**24**	**5**	**0**	**29**	**22**	**3**	**0**	**18**	**12**	**4**
Overall expected	**0**	**30**	**29**	**0**	**0**	**29**	**25**	**0**	**0**	**18**	**16**	**0**

However neither the one gall inoculum (Nassau, FL), nor the naturally existing inoculum in the field trial (Randolph, GA) was tested for avirulence. Yet, 0% of the *Fr1*/- clones from genotyped families 0 and 1 were diseased in either the one-gall trial or the field experiment, and 83% of the *Fr1*/*Fr1* clones from these families were diseased in the one-gall and field screens. This suggests that the inocula used in those screens were avirulent to *Fr1*, too. Furthermore, in an unpublished study varying the numbers of galls collected from counties; Santa Rosa, Madison, Alachua and Bradford, Florida virulence against *Fr1* was found to be very low in Florida (personal communication Dr Henry Amerson) (Figure 3-1).

There were a few cases where clones did not perform as anticipated. Four out of thirty clones in the ten-gall screen developed galls even though they were genotyped as *Fr1*/*Fr1* (Table 3-3). To investigate potential mislabeling problems needle samples from all the ramets belonging to these four clones were re-genotyped with markers AJ4 420 and J7 470 to ensure their identity. Based on the marker information, all plants were marker (-) for AJ4 420 and (+) for J7 470 confirming the genotypes previously assessed.

One possible explanation for the lack of marker-trait co-segregation in these cases was genetic recombination between the marker and *Fr1*. To evaluate the likelihood of this occurrence, I calculated the probability of recombination for both full sib families (0 and 1) and low values (9.3x10-6, 2.1x10-4 respectively) suggested that a recombination event between the marker and *Fr1* gene was extremely unlikely. On the other hand five out of twenty nine clones in the ten-gall screen, three out of twenty five clones in the one-gall screen and four out of sixteen clones in the field did not show disease although they were genotyped as *Fr1*/*Fr1* (Table 3-3). These were potentially examples of physiological "escapes" that are described in more detail in the next paragraph.

The Genetic Basis for "Escape Rate"

Clonal replication provided multiple observations of individual host genotypes and helped identify "escapes" (disease free ramets from a susceptible clone, Figure 3-2). Because most clones that were susceptible had at least one ramet was diseased (83 % of *Fr1*/*Fr1* clones fit this category; Table 3-3), I used all of the families from greenhouse and field screens in the analysis. In an idealized experiment in which the "escape rate" (ER) was zero, all ramets from a given clone would be either gall-free or galled. If a histogram is plotted illustrating the percentage of galled ramets for a given clone in an experiment without any escapes I would have two bars; one at 0 percent (resistant); and a second one at 100 percent (susceptible). To evaluate the distribution of percentage of galled ramets per clone in each screen, I plotted histograms for the ten-gall, one-gall and field tests (Figure 3-4). The existence of the bars at 20%-80% drew attention to the significance of escapes in these screens. This suggested a role for one or more environmental factors that prevented normal expression of the disease phenotype.

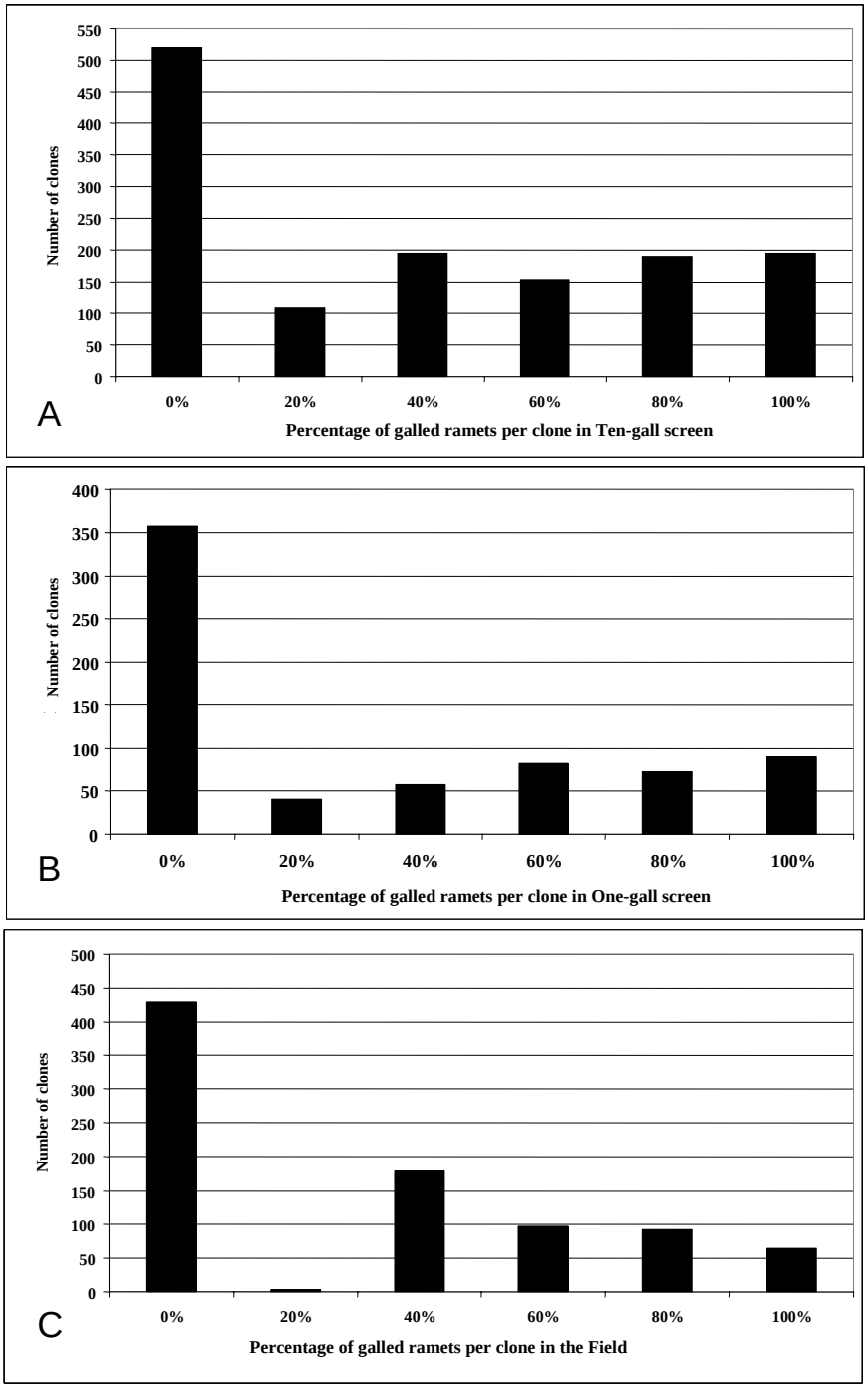

Figure 3-4 Distribution of percentage of galled ramets by clone in the ten-gall (A), one-gall (B) and field (C) screens. There were a total of 1471 genotypes (i.e. clones) in all the experiments and each clone was replicated 1-5 times in each experiment.

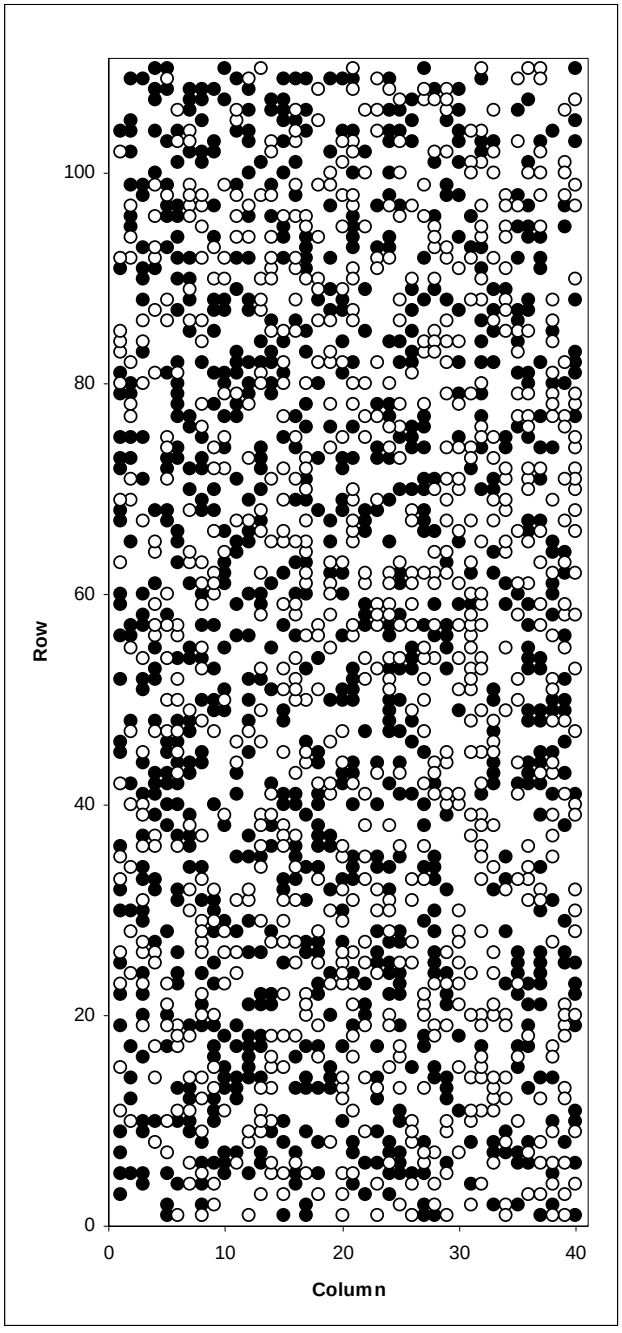

Figure 3-5 Random distribution of fusiform rust disease resistance performance of ramets from clones that had at least one diseased ramet in Randolph, GA field trial. Ramets that formed galls were illustrated as full circles whereas healthy individuals were presented as empty circles. Column and row refers to the exact location of each ramet as they were planted in the field. A lack of circle indicates the position of a ramet in a disease-free genotype.

ER might be an environmentally driven event or it might be under control of genetic factors. Replication of the clones as ramets and placing them randomly in different blocks enabled us to calculate escape rate for each clone and compute heritability for this trait. Clones with no galled ramets were not included in the analysis since genetic and physiological resistance cannot be distinguished in these cases. According to our calculations, ER was nearly as heritable as score (disease incidence) in both greenhouse screens (Table 3-2). This was consistent with the explanation that escape rate in the greenhouse was controlled in part by genes. In contrast, when I ran the field data for the same trait, the heritability was zero. Thus, ER was only heritable when the cuttings were in the greenhouse and it was non-genetically controlled in the field. I ruled out the possibility that infection occurred in a specific pattern (i.e. only on the north side) and show that infection was spread uniform in the field; I graphed the distribution of ramets from susceptible clones in the field (Figure 3-5). I also tested distribution of resistant and susceptible clones in field to find out if there was any non-random pattern to their placement in field area. Asymptotic Z-test results were not significant implying that the resistant and susceptible clones and ramets were distributed in field in a random fashion.

Discussion

In this study I utilized a large, structured population of loblolly pine that had been phenotyped in the greenhouse and in the field for resistance to fusiform rust disease, which is an endemic pathosystem in which specific resistance has evolved.

Agreement Among Greenhouse and Field Screens

Greenhouse disease screens are performed to predict resistance classes the genotypes will fall into in the field. In the greenhouse I can control nearly all conditions

whereas our influence on conditions in the field is limited to experimental design. One of the most important parameter in disease screen is the inoculum; C quercuum which has varying pathogenicity in geographically different field sites (Kuhlman, 1990; Powers and Langdon, 1977; Powers Jr, 1985; Snow et al., 1975; Snow and Kais, 1970; Walkinshaw and Bey, 1981). An implication of genetic variation at the pathogen side is unpredictable performance of elite crosses across field sites (McKeand et al., 2003). Furthermore, it was discovered recently that in the field, a single gall is usually induced by a single *C. quercuum* basidiospore (Kubisiak et al., 2004), whereas concentrated basidiospore spray system (CBS) inoculation allows multiple haplotypes to infect and form a single gall (Kubisiak et al., 2005). Thus, the host can be screened for resistance against different genotypes of virulent pathogen in a single experiment and resistant genotypes would perform more consistently across the sites. In this study I not only conservatively score a clone as susceptible if it had at least one ramet diseased but I also screened both in the greenhouse and the field. This cautious approach aided us in identification of resistant individuals with complete penetrance of disease resistance trait. Thus, concentrated basidiospore spray system I used to inoculate the clonally propagated material in RSC and transformation of continuous data (clonal means) to binary scale (0< ramets diseased: susceptible, 0=ramets diseased: resistant) conservatively predicted resistance/ susceptibility of the genotypes I was testing.

I found that greenhouse and field data showed high genetic correlation, presumably due to genetic similarity of the inocula utilized in the greenhouse screen and the inoculum in the natural ecosystem at the field site. These results support the earlier reports where gall score in the field yielded similar heritabilities as greenhouse screens (de Souza et al.,

1990; Miller, 1983). Moreover the genetic correlation between greenhouse screens and the field trial was very high suggesting greenhouse screens can be used to select elite parents, to breed for fusiform rust resistance in the field. Fusiform rust rankings from the Randolp, GA field site was obtained from 2nd year data which would be considered as "preliminary" for this disease. Multiple field site data would be available in near future and then I can compare the field fusiform rust rankings with the ones I calculated from the greenhouse screens.

Marker-trait Cosegregation for Fusiform Rust Disease Resistance

Markers segregating with resistance genes have been used for selection purposes over the last decade (Francia et al., 2005). This kind of information recently became available for the fusiform rust-loblolly pine pathosystem (Wilcox et al., 1996). Host genotyping with markers linked to the known pathotype-specific resistance gene *Fr1* revealed marker-trait cosegregation in both greenhouse and field screens. I used this RAPD marker data to predict performances of clones from the two families which were segregating for *Fr1* resistance gene. The ten-gall inoculum used in the greenhouse experiment was tested for virulence towards *Fr1* before this screen and found to be avirulent (Avr) since after the challenge with this inoculum the clones with *Fr1*/- (resistant) genotypes were gall-free and *Fr1*/*Fr1*(susceptible) genotypes were mostly diseased (personal communication Dr Henry Amerson). In the ten-gall screen clones that were genotyped for *Fr1* performed as expected from their genotypes, confirming the previous results. Cosegregation data in the one-gall screen showed that *Fr1*/- genotypes were disease free and *Fr1*/*Fr1* genotypes were mostly diseased, these results suggested that the one-gall inoculum was also avirulent to *Fr1* resistance gene. I did not have control over inoculum in the field as I did in the greenhouse screens. However, the data

collected in Randolph, GA on families 0 and 1 indicated that the marker was holding up quite well. Thus, all the inocula I used for these screens were avirulent to *Fr1*.

I identified some clones that were genotyped as *Fr1*/- but expressed a susceptible phenotype. I initially considered that these may have been cases in which *Fr1*/*Fr1* genotypes were mislabeled as *Fr1*/-. I repeated the genotyping reactions on diploid tissues of diseased cuttings and verified that all of ramets for each of the 'unexpected' classes of genotypes (Table 3-3, grey boxes) gave rise to the same genotypic classes that were assigned to them based on megagametophyte genotyping. Hence I favor the explanation that these exceptions to marker-trait cosegregation are due to a low level of virulence in the inoculum, i.e., a low frequency of basidiospores with virulence to *Fr1*. Consistent with this view is the observation that all four cases of diseased *Fr1* genotypes in the ten-gall screen were due to single diseased ramets (data not shown). Moreover ten-gall inoculum was a mixture of ten galls from a high hazard site; also the inoculation load in greenhouse was much higher than in the field. Diseased *Fr1*/- clones could be the result of a recombination event between the marker J7_485A and the actual resistance gene *Fr1*; however, our calculations show that the likelihood of having a recombination between the marker and the resistance gene in the families 0 and 1 was very low, thus unlikely. Thus, a much more likely explanation for these cases of disease in the presence of the DNA marker was that these four genotypes were infected with virulent *C. quercuum* genotypes that were present at relatively low frequency in the ten-gall inoculum.

Penetrance of the Fusiform Rust Disease Phenotype

Clonal propagation enabled us to quantify the penetrance of the fusiform rust disease phenotype in genotyped and non-genotyped families within the structured

population. Although the disease phenotype was expressed at a similar frequency in both greenhouse and field screens – as revealed by similar proportions of galled ramets and clones – the penetrance of the disease phenotype was dramatically reduced in both greenhouse screens relative to the field. The basis for this conclusion is that escape rate (the lack of disease symptom development in a susceptible host genotype) was heritable and similarly so in magnitude across both greenhouse screens, but not heritable in the field. The biological explanation for the reduced penetrance of the disease phenotype in the greenhouse could be driven by pathogenic variation; there may be a low level of avirulence in the ten-gall and one-gall inocula that correspond to unmonitored host resistance genes that are segregating in the structured population. If this explanation is correct, then the ten-gall and one-gall inocula must harbor similarly low levels of avirulent pathotypes such that equivalent heritability estimates are obtained, and the heritability of escape rate (ER) is being driven by segregation of resistance genes in the structured population. Alternatively, the biological explanation for the reduced penetrance of the disease phenotype in the greenhouse could be driven by host physiological genetics; there may be inconsistent growth (shoot flush) rhythms among genotypes that lead to a lack of infection in some genotypes at the time of inoculation. If this model is correct, then the heritability of ER is being driven by segregation of genes that directly or indirectly regulate production of shoot tissues that are potential infection courts. Distinguishing between these competing models should be feasible when sufficient marker coverage allows association testing between candidate genes and disease phenotypes (Brown et al., 2004).

Pathogen Infection in an Ecologically Relevant Setting

Certainly there were many differences in both host and the pathogen dynamics in the greenhouse compared to the field. Because the artificial inoculation with rust spores in a greenhouse screening trial normally occurs within a narrow window of time (Knighten, 1988), all ramets may not have succulent shoots that are susceptible to the pathogen at that time. This phenomenon might stem from the fact rooted cuttings harden off faster than seedlings, preventing an otherwise successful infection. *C. quercuum* prefers young actively growing plant tissue to infect (Griggs and Schmidt, 1977), so a genetically susceptible ramet might not be convenient for infection if it already had hardened off. In contrast, field-grown trees may be exposed to inoculum periodically over a much longer time span of several weeks after spring (Schmidt, 1998), so it is much more likely for the pathogen to find the host in a succulent state. Random distribution of more favorable micro sites with more water, fertilizer and sunlight likely affected the escapes in the field. This approach reinforced the conclusions of previous reports (Foster and Anderson, 1989; Frampton et al., 2000; Stelzer et al., 1998) where rooted cuttings became physiologically equivalent to the seedlings after several years in field and had higher resistance to fusiform rust then the seedlings. It is very likely that these changes in both host and the pathogen lead to loss of heritability of escape rate in the field.

I also investigated the possibility of a non-random dispersion of basidiospores on field. However, I did not find any evidence to support uneven dispersion of inoculum over the field which would result in spatial pattern of susceptible ramets suggesting a driver of zero heritability. The diseased plants were scattered all over the field site in a random fashion. Thus, the lack of ER heritability in the field was not a consequence of

spatially nonrandom infection. I surmise that extended periods of inoculation may occur under natural conditions, obscuring genetic influences of host shoot phenology on ER.

In this study I examined the architecture of fusiform rust disease resistance in a large structured population in which I considered resistance as a binomial trait (i.e., resistant or susceptible) for each host genotype. This is in contrast to other studies in which disease resistance was scored on a continuous scale for each genotype, based upon the proportion of ramets that exhibited disease symptoms (Frampton et al., 2002; Isik et al., 2004; Kayihan et al., 2005). Both approaches have value, based on objectives; evaluating resistance at clonal level might aid answering biologically important questions about disease inheritance whereas the clonal mean for disease incidence approach might reveal quantitative genetically important questions such as the amount of epistasis. Both approaches have reduced amount of error with the use of clonal propagation. Moreover, the existence of these clonally replicated field trials presents an opportunity to monitor potential shifts in pathogen virulence that may occur in the *C. quercuum* population. Such shifts may occur in part due to increased planting of resistant genotypes in the field, and create potentially greater risks if planting stock is clonal.

CHAPTER 4
TRANSCRIPT PROFILING REVEALS POTENTIAL MECHANISMS OF FUSIFORM RUST DISEASE DEPENDENT SHIFTS IN PINE STEM DEVELOPMENT

Introduction

Loblolly pine is one of the most economically important tree species in the southeastern United States since loblolly pine plantations cover nearly 13.4 hectares (Schultz, 1999) in this region and over 1 billion seedlings are planted annually (McKeand et al., 2003). These plantations have been threatened by the endemic fungus *Cronartium quercuum* Berk. Miyable ex Shirai f. sp. fusiforme (Cqf) (Burdsall and Snow, 1977) which incites fusiform rust disease. Fusiform rust is one of the most destructive fungal diseases in the South causing damage in millions of dollars every year (Cubbage et al. 2000).

Cqf is a biotrophic fungus that induces gall formation on susceptible trees. The pathogen causes a number of abnormal changes in the stem – the galls themselves have an organized cellular structure distinct from a normal stem when viewed using light microscopy. The Cqf hyphae are intimately associated with cortical cells phloem and xylem ray cells, and with cambial cells, with the hyperplasia (swelling) of the stem apparently due to an increase in the number of xylem ray cells and vertical resin ducts in the diseased stem relative to the healthy tissue (Jackson and Parker, 1958). There is evidence that galls disrupt water transport in diseased trees based on nuclear magnetic resonance imaging of galled and healthy stems (MacFall, 1994) and expression of desiccation-associated genes in galled vs. healthy stems (Myburg et al., in press). Galls

weaken the structural integrity of stems such that diseased trees are more susceptible to breakage by wind, resulting in reduced stocking in stands (Cubbage et al., 2000). Thus the galls alter both structural and functional features of pine stems.

The interactions between the host and the pathogen during gall formation are far from being completely understood in the loblolly pine-fusiform rust system. However recently, transcript enrichment techniques (Warren and Covert, 2004) and microarray analysis have begun to identify genes from both pine and Cqf that are differentially expressed at infection, gall initiation and gall expansion stages (Myburg et al., in press). The Myburg et al. (in press) study is of particular relevance to this chapter, in that I have re-analyzed the data presented in Myburg et al. to extract information on the expression profiles of differentially regulated genes. The Myburg et al. paper presented the overall study design and the analysis focused on contrasts between selected time points to identify genes with potential roles in specific stages of gall development (i.e., infection, initiation and expansion). In this study, I focused on the actual expression profiles of individual genes, which I define as the observed change in transcript abundance across time intervals. This analysis allowed me to identify genes whose profiles differed by treatment (pathogen vs. control), genotype (resistant vs. susceptible) and disease state (diseased vs. healthy).

Materials and Methods

Plant Material, Genotyping and Harvesting

The genetic materials, fingerprinting and sample collection methods are described in a previous study (Myburg et al. in press). Briefly, seedlings from the cross (10-5 ♀ × 4666-4 ♂) segregate for *Fr1* because it is a testcross between genotype 10-5, which is heterozygous for the dominant resistance gene *Fr1* (Wilcox et al., 1996), and 4666-4

(*Fr1*/*Fr1*). The megagametophytes were harvested from each germinating seedlings and the haploid tissue was screened for RAPD markers J7_470 (Wilcox et al., 1996) and AJ4_420 that define a ~1 cM interval containing *Fr1*. The RAPD markers identified 350 resistant (*Fr1*/*Fr1*; +J7_470, -AJ4_420) and 350 susceptible (*Fr1*/*Fr1*; -J7_470, +AJ4_420) individuals.

Among the 350 individuals in each resistance class 210 individuals (15 seedlings x 2 biological reps x 7 time points) were challenged with Cqf and 140 individuals (10 seedlings x 2 biological reps x 7 time points) were inoculated with distilled water (as control). Twenty additional seedlings were water inoculated and included in the study as index plants. These index plants were marked with two black ink spots, one immediately below the apical bud and the other approximately 1.5 cm below the first spot where the potential gall formation with take place. Using the references from the index plants, tissue from this region was harvested before the onset of visible disease symptom. The first harvest time point was 90 min after inoculation followed by additional harvests at 6hrs, 24hrs, 7 days, 28 days, 56 days and 112 days post inoculation.

Fungal Material and Inoculation

A single aecisospore isolate of *C. quercuum* that was avirulent to *Fr1* (SC 20-21, obtained from E.G. Kuhlman, USDA-FS, retired) was used in the inoculations that were performed at the Resistance Screening Center, Asheville, NC following their standard inoculation protocol except inoculum was increased by 400% to minimize escapes.

Microarray

Experimental design, microarray preparation, target synthesis, microarray hybridization and scanning were described in Myburg et al. (in press).

Statistical Analysis

The experiment was implemented in a balanced incomplete block design (Kerr and Churchill, 2001) and analyzed using a mixed model approach (Wolfinger, 2001). In order to compare the gene expression on several arrays treated with RNA from different treatment X genotype combinations that were dyed with 2 different dyes I applied a normalization data to the entire dataset. The normalization model was chosen with respect to the significance of the effects that extracted from the full model where every effect and the combinations of effects were tested. I assumed dye, genotype, time and treatment did not change the overall level of gene expression in a single biological sample. The resulting normalization model for the log2 transformed data (yijklm) was:

$$y_{ijklm} = \mu + a_i + D_j + G_k + T_l + M_m + D * M_{jl} + M * T_{lm} + e_{ijklm}$$

where:

μ is the population mean.

a_i is the random variable array ~NID(0,σ^2_a), i=1–56

D_j is the fixed resolvable dye, j=1–2

G_k is the fixed resolvable genotype, k=1–2

T_l is the fixed resolvable treatment, l=1–2

M_m is the fixed resolvable time, m=1–6

$D*M_{jm}$ is the fixed resolvable dye by time

$T*M_{lm}$ is the fixed resolvable treatment by time

e_{ijklm} is the random variable error within the experiment ~NID(0, σ^2_e).

Before residual values derived from this model were incorporated into the gene-specific model I deleted the two early time points (1.5 hrs and 6hrs) since any of the three

time points within a 24 hr time period should represent the stage of stem development at the time of the inoculation. The multiple early sample times were initially chosen to try and capture gene expression shifts associated with a rapid hypersensitive response; however this was unsuccessful (Myburg et al., in press). I chose to retain the 24 hr time point since the inoculation procedure involves incubation of trees in a humid chamber for 6-12 hr, whereas the 24 hr (hereafter referred to as "1day"), 7 day, 56 day and 112 day samplings were all carried out in the greenhouse. The 1 day, 7 day, 56 day and 112 day data were then analyzed using the gene model:

$$y_{ijklm} = \mu + a_i + s(a)_{ij} + G_k + T_l + M_m + G*M_{kl} + M*T_{lm} + G*T_{kl} + G*T*M_{klm} + e_{ijklm}$$

where:

μ is the population mean.

a_i is the random variable array ~NID(0, σ^2_a), i=1–56.

$s(a)_{ij}$ is the random variable spot number(array) ~NID(0, $\sigma^2_{s(a)}$, j=1–4.

G_k is the fixed resolvable genotype, k=1–2.

T_l is the fixed resolvable treatment, l=1–2.

M_m is the fixed resolvable time, m=1–4.

$G*M_{km}$ is the fixed resolvable genotype by time.

$T*M_{lm}$ is the fixed resolvable treatment by time.

$T*M_{klm}$ is the fixed resolvable genotype by treatment by time

e_{ijklm} is the random variable error within the experiment ~NID(0, $\sigma^2_{e)}$.

I used PROC MIXED in SAS (SAS Institute Inc. SAS/STAT Software version 9, SAS Institute, Cary, NC) to run both the array and gene level models (Wolfinger et al., 2001) (Figure 4-1). I identified genes that were significant for genotype, treatment, time,

genotype by time, genotype by treatment, time by treatment and genotype by time by treatment using the ANOVA F-test combined with Bonferroni multiple testing correction (p=0.01). Using this conservative criterion for significance, a total of 861 genes were significant for one or more of these effects.

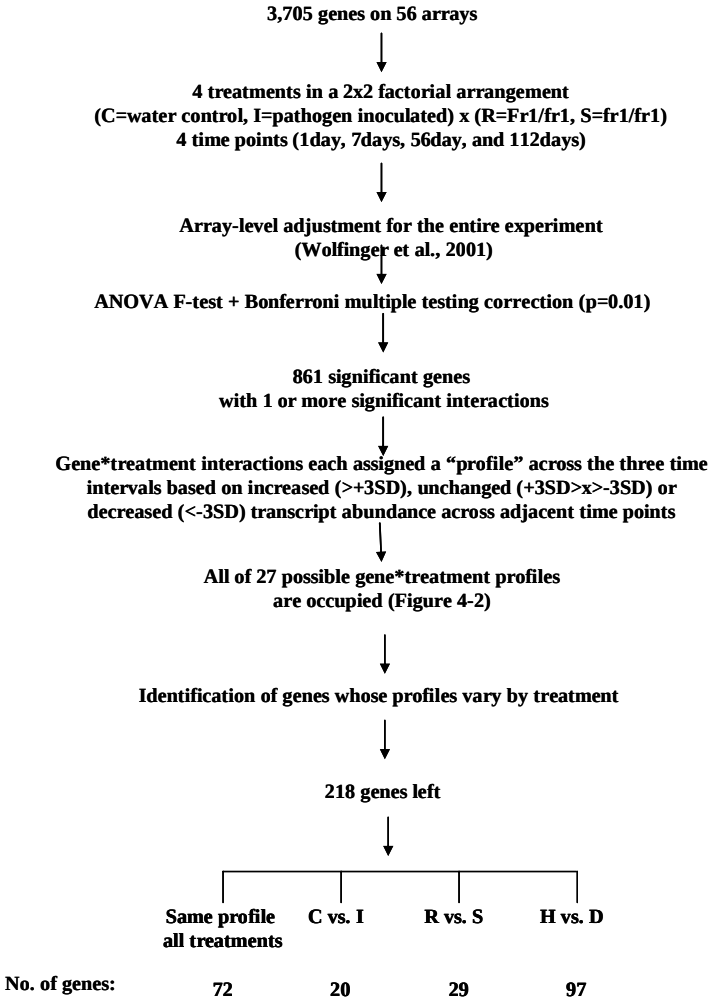

Figure 4-1 Flow chart illustrating the procedure to identify significant and biologically interesting gene expression profiles. ANOVA was performed for each of the 3705 genes. After experimentwise correction for multiple testing, significant gene*treatment interactions were assigned "profiles" comprised of three time intervals (1d to 7d; 7d to 56d; 56d to 112d) based on shifts in the LS mean for transcript abundance at each adjacent time point. Biologically interesting profile contrasts are explored; "SI vs. rest" is equivalent to "diseased vs. healthy."

I established profiles of transcript abundance that these 861 genes followed on the four time points (i.e. three intervals) (Figure 4-1). To establish these profiles I began with the least square mean for each significant gene in each treatment and time point. Using the "pdiff" option I identified mean differences and standard deviations associated with the mean differences, for each consecutive time point within each treatment. At any given time increment (e.g between 1 day and 7 days), expression of a gene can increase, decrease or not change. A decision rule was applied in which expression for a gene was declared "not changed" if the absolute mean difference between the adjacent time points was within 3 standard deviations. However if the absolute mean difference of gene expression was greater than 3 standard deviations within a time increment it was declared "increased" or "decreased." I simply joined the three adjacent time intervals for a gene in order to assign it a "gene profile." A gene profile is thus comprised of three integers representing the change in gene expression across the three adjacent time intervals in the experiment. For example, a gene that increases in expression at each interval has a profile 1 1 1, whereas a gene that decreases during the first interval and does not change thereafter has a profile -1 0 0.

Results

Among 3705 genes that were evaluated for gene expression, 861 genes were significant for time, genotype, treatment, time*treatment, treatment*genotype, time*genotype, or time*genotype*treatment with Bonferroni corrections for multiple testing (p=0.01) (Figure 4-1). Since there were only 3 time intervals and 3 possible trends within each interval, there can be only 27 distinct profiles. The diversity of profile types among significant genes was sufficiently high to occupy all 27 possible profile groups. I

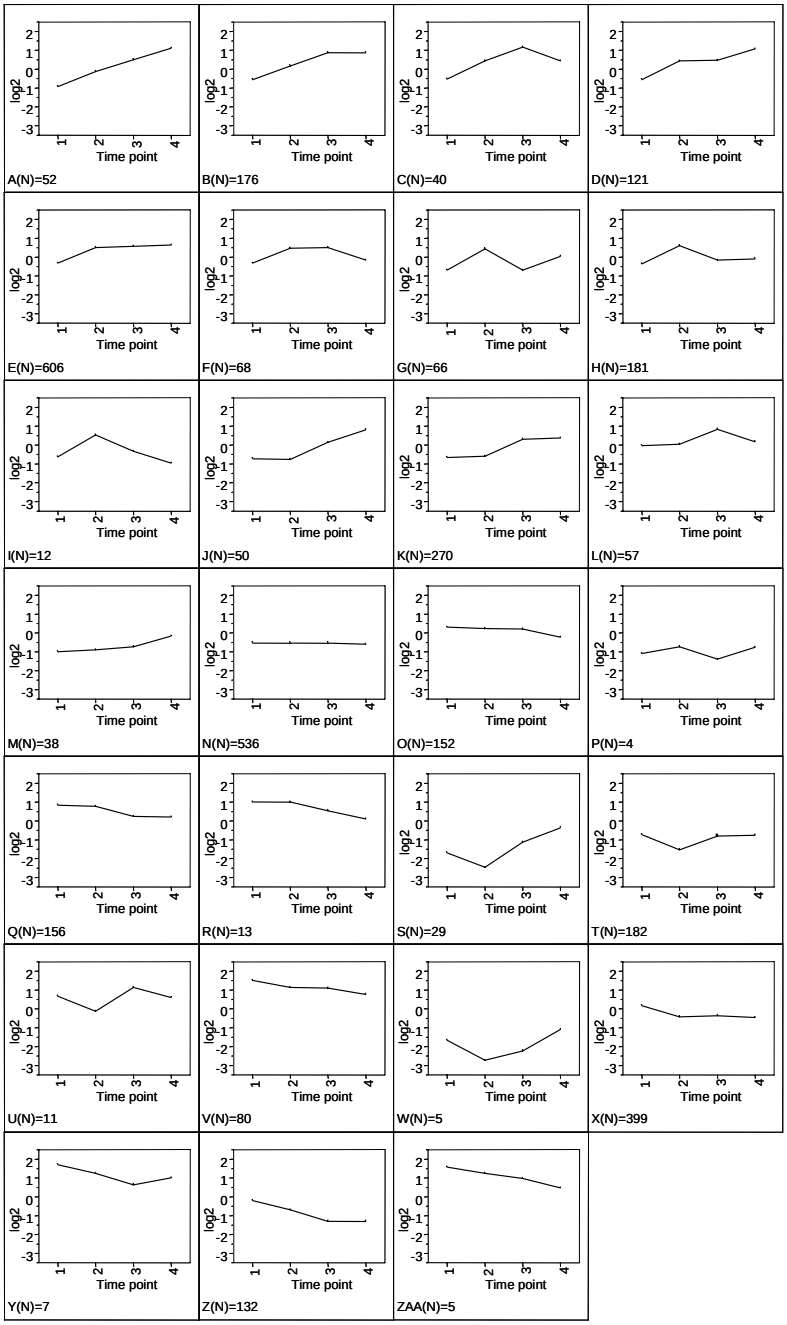

Figure 4-2. Analyses of mean gene expression data support 27 distinct profile groups, "A" through "AA." The genes that were significant for time, genotype, treatment, time*treatment, treatment*genotype, time*genotype, or time*genotype*treatment (p=0.01) were categorized into 1 of 3 possible profiles (i.e. up, down or unchanged) based on variation 3 standard deviations from the mean. Mean gene expression (y-axis) was plotted across the time intervals (x-axis) for each profile group. Time points are indicated as 1=1 day, 2=7 days, 56 days=3 and 112 days=4. N= the number of gene or gene interactions that fall into a particular profile group.

described these profile groups and counted the gene–treatment combinations falling into these groups (Figure 4-2). The frequency at which non-regulated gene and gene interaction profiles were detected ("N"; N=536), however the number of genes that were unregulated in all four treatments was much lower (N=5 genes; data not shown).

The criteria I used to distinguish profiles allowed me to investigate the trends of a given gene expressed in resistant-control (RC), resistant-inoculated (RI), susceptible-control (rC), susceptible-inoculated (rI) treatment conditions. There were 72 genes that did not respond differently to the genotype-treatment combinations; in other words, the profile of gene expression was not affected by genotype or by fungal inoculation (Figure 4-3).

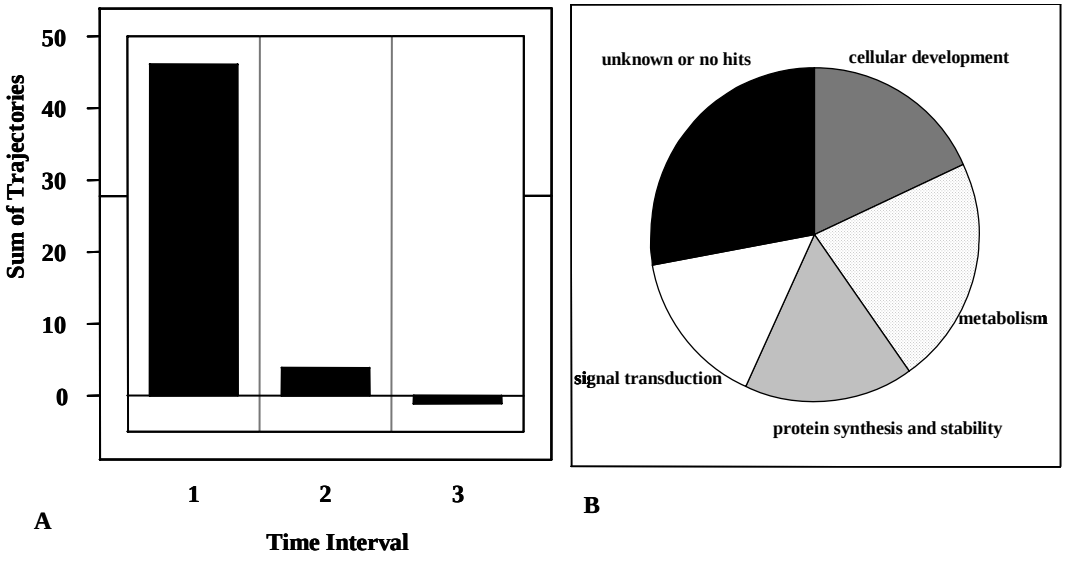

Figure 4-3. Genes with the same expression profile in all treatment combinations were predominantly induced during the first time interval. (A) For each time interval, profiles were summed across the significant genes and plotted. Time interval 1=interval from time point 1 (1d) to time point 2 (7d), interval 2=interval from time point 2 (7d) to time point 3 (56d) and interval 3=interval from time point 3 (56d) to time point 4 (112d). (B) Chart of the genes significantly up-regulated in the first time interval but unchanged with respect to treatment or genotype after categorization into functional groups.

As stated before each gene was assigned a series of numbers (1, 0 or -1) to describe the trend within a treatment. The sum of these numbers (across genes) for a given time interval illustrates the profile of overall expression for those genes (Figure 4-3A). According to this analysis the genes that had the same profiles in all treatment combinations were predominantly up-regulated within the first time interval (1 day-7 days). For the rest of the time intervals the expressions of the 72 genes were nearly equally up and down regulated. There were 41 genes with a profile in which expression increased in interval 1 (Figure 4-3 B); these genes were in category "E" for all gene-treatment combinations (Figure 4-2).

I extended this analysis to identify potentially interesting genes whose profiles changed according to treatment (Figure 4-4). Genes significantly regulated between "control" vs. "inoculated" treatment classes differed mainly in the 3rd interval (56-112 days) (Figure 4-4A). In contrast, genes regulated between "resistant" vs. "susceptible" genotypic classes differed mainly in the 1st time interval (1-7 days) (Figure 4-4B). In the comparison of "healthy" (resistant-inoculated, resistant-control, susceptible-control) vs. "diseased" (susceptible-inoculated) classes (Figure 4-4C) the 2nd time interval (7-56 days) was the interval during which most genes were regulated. Among these biologically interesting comparisons only the healthy vs. diseased contrast revealed genes of fungal origin. There were 13 known fungal genes regulated in this category – most of these occurring in the second time interval (7days-56 days) – with possible additional fungal genes classified as "unknown" origin (Figure 4-4C).

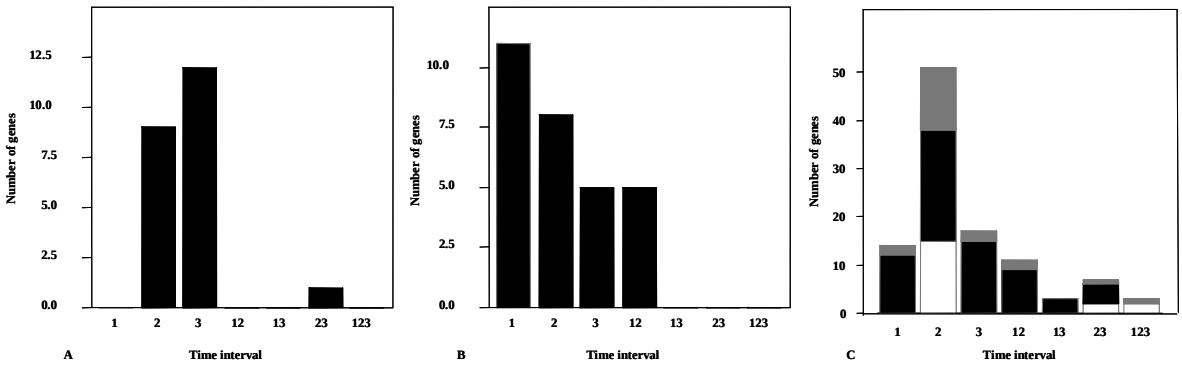

Figure 4-4. Profile groups can be categorized into biologically interesting clusters with distinct changes in gene expression patterns. Genes significantly regulated across "control" and "inoculated" treatment classes (A), across "resistant" and "susceptible" genotypic classes (B) and across "healthy" (resistant-inoculated, resistant-control, susceptible-control) and "diseased" (susceptible-inoculated) classes (C) are shown. Time intervals 1, 2, and 3 are as defined in Figure 2. 12 = intervals 1 and 2 combined, 23= 2 and 3 combined, 13= 1 and 3 combined, and 123= 1, 2 and 3 combined. The genes that originated from host are represented by a black bar, genes with no known origin are represented by a gray bar and fungal genes are represented by a white bar.

I identified genes that were differentially regulated in diseased vs. healthy tissues, and found there were dramatic shifts in all three intervals. The three intervals had been previously characterized as reflecting distinct phases of disease development, specifically the 1st time interval = infection, 2nd time interval = gall initiation and 3rd time interval = gall expansion (Myburg et al., in press). I contrasted the profiles in diseased vs. healthy tissues in order to identify the direction of regulation imposed by the pathogen (Figure 4-5). In the "infection" interval, most of the genes that are differentially regulated were down-regulated in diseased seedlings. During gall "initiation" the majority of the genes were up-regulated and 11 of the 50 genes that were in this class were fungal genes whereas 27 of them were pine genes and the rest (12 genes) were unknown. The "gall expansion" phase was dominated by pine genes, with half of the genes in this class up-regulated and the other half down-regulated under the influence of Cqf.

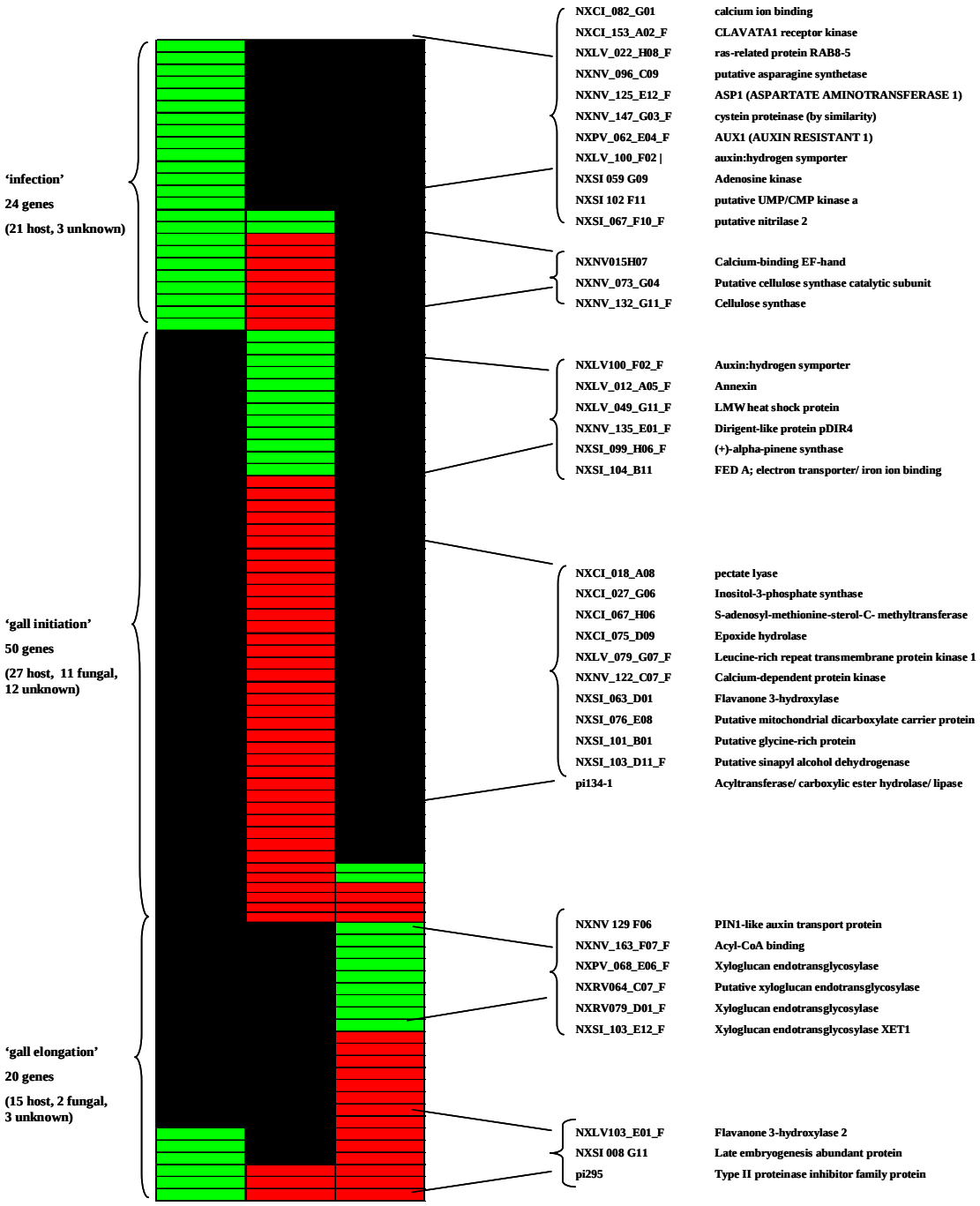

Figure 4-5 Clustergram of gene profile differences (or contrasts) between diseased and healthy treatments. Genes whose expression in diseased tissues are higher than in healthy tissues for a given interval are shown in red; if lower, green; if identical, black. Examples of genes in selected clusters are shown on the right.

The genes regulated during the "infection" phase are similar to genes that encode proteins involved in auxin transport (NXLV_100_F02 = auxin:hydrogen symporter;

NXPV_062_E04 F = AUX1 auxin transporter) and auxin biosynthesis (NXSI_067_F10F = nitrilase 2, an IAA biosynthetic gene). The "infection" phase also appears to involve calcium fluxes as indicated by regulation of genes involved in calcium signaling (NXCI_082_G01 = calcium ion binding protein). During gall "initiation" cell wall modification enzymes appear to be induced under the influence of Cqf (NXNV_132_G11F = cellulose synthase; NXCI_018_A08 = pectate lyase; NXSI_101_B01 = putative glycine-rich protein; NXSI_103_D11F = putative sinapyl alcohol dehydrogenase). During the gall "expansion" phase, four distinct members of the xyloglucan endotransglycosylase family were down-regulated, potentially in association with additional cell wall architecture modifications during gall growth (NXSI_103_E12F; NXPV_068_E06F; NXRV064_C07_F; NXRV079_D01F = xyloglucan endotrans-glycosylase).

Discussion

Cronartium quercuum is a biotrophic, macrocyclic, heteroecious fungus (Burdsall and Snow, 1977) that incites abnormal changes in the fusiform rust susceptible pine stem such as swollen phloem cells, and increase in the number of resin ducts and ray cells (Jackson and Parker, 1958; Gray and Amerson, 1983, Jewell et al., 1962; Miller et al., 1976). To identify genes and processes that may underlie the development of disease symptoms, I used a microarray dataset derived from a time course analysis of fusiform rust disease development. The genes on the microarray included ESTs and cDNAs obtained from subtraction libraries and from genes that are expressed during the interaction between the host and the pathogen, an approach that has been successful in identifying genes that may condition disease phenotypes (Birch and Kamoun, 2000; Wan et al., 2002). These powerful tools became available for the fusiform rust-loblolly pine

pathosystem recently (Warren and Cover, 2004; Myburg et al., in press). In this study I investigated fungal and pine gene expression on the pine stems obtained from resistant and susceptible seedlings that were inoculated with Cqf or water using an experimental design that involved 4 time points. I captured a diverse array of expression profiles across the genes that were significant for the main effects or interactions of the main effects I was testing.

Transcription Profiling Reveals Differential Gene Expression

Diverse patterns of gene expression were observed in this study, such that all possible combinations of 27 profiles were occupied by at least one gene or gene interaction. The chip-level model that I used, effectively corrected for statistically significant treatment and interaction effects at the chip level prior to the gene-level analysis. This approach assumes that large-scale unidirectional shifts in gene expression in any particular treatment or treatment combination are based on technical artifacts, not biologically meaningful effects, in the microarray experiment. A chip-level adjustment model with fewer terms may be more biologically appropriate for this study, in that it may identify more genes whose expression is altered; on the other hand, such an approach may lead to more false positives. The decision to use 3 SD as a criterion for significance was based on striking a balance between identifying potentially interesting biological mechanisms, while still being sufficiently conservative to exclude most false positives. An indication that this criterion was reasonably conservative was the observation that although all of the gene and gene interactions were highly significant experimentwise (after Bonferroni correction), 648 profiles were declared biologically non-significant based on the 3 SD criterion.

There were 72 genes that showed identical profiles regardless of treatment (genotype or pathogen). The majority of the genes in this category belonged to group “E” which is characterized by an increase in interval 1 and stable expression thereafter. The 1 day to 1 week period of pine seedling growth during this study is predicted to be a time of active primary stem growth and development. The annotation of the genes in group “E” for all treatments revealed that they belonged to functional groups such as metabolism, protein stability, signal transduction and cellular development, which is consistent with the kinds of functions expected in an actively growing seedling. Notably, these genes are not affected by genotype or by pathogen challenge, so they are more relevant to stem development in loblolly pine than to disease development per se. Such genes presumably reflect the juvenile developmental state of the stem when the seedlings were harvested at the earliest time point in the experiment.

When the transcriptomes of control and pathogen inoculated seedlings are compared, one might expect that the difference in gene expression would be at the first time interval since that would be the period during which pathogen spores are germinating and contributing to the transcriptome (this might occur on both resistant and susceptible seedlings). Interestingly, this analysis suggests otherwise, in that the effect of Cqf inoculation is on the regulation of genes much later than the first time interval. In fact, the effects of Cqf inoculation on gene expression profiles gradually increased through the 3rd time interval. Thus, both resistant and susceptible plants gave the same response to a fungal infection attempt that resulted in differences between the control and the inoculated plants. It is intriguing to speculate that some kind of pathogen-induced systemic response might be responsible for this observation. However, known examples

of long-term pathogen-induced responses (such as systemic acquired resistance and induced systemic resistance) are typically incited by an incompatible interaction (resistant host) distinct from the compatible interaction (susceptible host), which is not consistent with my findings. In addition, the number of genes in this group was relatively small (N=20), so further gene expression studies should be performed to confirm and resolve this phenomenon.

The comparison of profiles in resistant vs. susceptible genotypes revealed differential gene expression, regardless of whether the plants were inoculated with Cqf or the water control. This is interesting in and of itself, since it suggests that allelic differences at the *Fr1* locus (and loci linked within 1 cM of *Fr1*) can be detected at the level of gene expression. The level of genetic resolution in this study was reasonably high, in that flanking markers were used for genotyping (thus only double recombinants within a 1 cM interval would be misclassified), and a relatively large number of seedlings was bulked for each time point (50 *Fr1*/*Fr1* and 50 *Fr1*/*Fr1* seedlings for each time point). Therefore the gene expression comparisons at each time point are likely to reflect allelic differences at or near *Fr1* but mixtures of both alleles at unlinked loci. One would expect that regulation could occur in cis- or in trans-. If cis-regulation is being observed here, then markers within the regulated genes could be used to create a fine map of loci in the *Fr1* interval. If trans-regulation predominates (e.g., Kirst et al. 2005), then allelic configuration at or near *Fr1* may induce downstream signaling mechanisms that are manifest on the microarrays.

Influences of Fusiform Rust Disease Development on Gene Profiles

The healthy vs. diseased comparison revealed over a hundred genes that were differentially regulated across all time intervals. In this section, I elaborate on the types of

putative gene functions and physiological mechanisms that may be involved in the development of the fusiform rust disease state.

In the first interval, a dramatic (down-) regulation of host regulatory genes and auxin biosynthesis and transport genes, in the developing disease state. Nitrilase 2 is the enzyme that catalyzes the conversion of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) which is active auxin (Woodward and Bartel, 2005). The auxin:hydrogen symporter is an efflux auxin carrier whereas AUX1 is an influx auxin carrier. Therefore, in diseased seedlings it is feasible that auxin transport as well as biosynthesis is impaired (or its transport is modified under the influence of the pathogen) within infected cells. Ca+2 is a well-known second messenger acting downstream of many stimuli including hormone signaling, and specifically in auxin signaling since calmodulin binding proteins are encoded by members of the auxin response gene family (SAURs; Yang and Poovaiah, 2000). Interestingly, during gall expansion there is a similar, coordinated regulation of a putative auxin transport protein-encoding gene (Pin1-like) and a gene encoding a protein containing a putative calcium-binding EF-hand. This provides another potential connection between auxin and calcium signaling in fusiform rust disease development. A potentially fruitful area for future research would be to quantify hormones in developing fusiform rust galls, since there is also evidence for a potential role of gibberellins in later stages of gall development (Myburg et al., in press).

In the second interval, transcripts encoded by the fungal pathogen were detected. The detection of fungal transcripts presumably reflects the establishment of a compatible interaction with the host (Heath, 1997) whereby the pathogen has begun to disperse within the host tissue (Walkinshaw, 1978) to manipulate the host sink to its needs. Since

Cronartium spp. are ecologically and economically important pathogens on pines (for example, the white pine blister rust is incited by *Cronartium ribicola* J. C. Fisch; Jurgens et al., 2003; Hudgins et al., 2005) there would be value in using transcript profiling approaches to better understand gene expression shifts in the pathogen component of the disease interaction in the future.

In the second interval I also observed disease-altered profiles of host genes potentially involved in modifying cell wall architecture. Cellulose synthase and pectate lyase were shown to be up-regulated in xylem (compared to leaves) (Paux et al., 2004), and in Populus reaction wood (Andersson-Gunneras et al., 2006). Sinapyl alcohol dehydrogenase is involved in the monolignol precursor pathway leading to lignin biosynthesis (Anterola et al., 2002) and glycine-rich proteins are thought to be involved in structural integrity and inducible reinforcement of plant cell walls (Ringli et al., 2001). Thus, host genes related to cell wall synthesis may be induced as a suite of genes required for rapid cell wall biosynthesis associated with the initiation of a gall.

In the third interval I observed regulation of a multigene family whose products are involved in growth and cellular architecture (loosening and/or tightening of the cell wall). Cellulose microfibrils are typically stabilized by xyloglucan moieties, which can be cleaved via xyloglucan endotransglycosylase (XET) so that cell can alter its shape (Fry et al., 1992). One of the family members is induced in the gall expansion phase, whereas four members are repressed, suggesting the family members have non-redundant roles in cell wall modification. It is interesting to note the rapid yet organized manner in which specific cell types in fusiform rust galls develop – ray cells increase in size and number, whereas resin ducts increase in number only (Jackson and Parker, 1958) - whereas the

attachments among parenchymal cells appear to loosen (Walkinshaw, 1978). Given the complex dynamics of cell architecture changes, it is tempting to speculate that some of the XETs may play roles in specific cell types and thus be performing distinct functions accordingly.

These results validate the stages of disease development proposed by Myburg et al. (in press) that seedlings can be analyzed according to the discrete disease phases of infection (1-7days), gall initiation (7-56 days) and gall expansion (56-112 days).

CHAPTER 5
CONCLUSION

Throughout this study I investigated two fungal diseases that are threatening loblolly pine plantations in southeastern US. The first disease is fusiform rust which is incited by biotrophic fungus *Cronartium quercuum* (Burdsall and Snow, 1977), the second one is pitch canker which is incited by the nectrorophic fungus *Fusarium circinatum* (Nirenberg and ODonnell, 1998). Biotrophic and necrotrophic fungi have different life cycles and infection mechanisms. Thus, a resistant host would respond differently to these two fungal diseases. A fusiform rust resistant host would recognize *C. querc*uum induce HR whereas *F. circinatum*, the pathogen can survive HR based defense by detoxification which is a common protection for necrotrophic fungi (Mayer, Staples, and Gil-ad 2001). Another major difference between *C. quercuum* and *F. circinatum* is the fact that *F. circinatum* requires wound to enter the host (Kuhlman 1987) while *C. quercuum* does not. Another host defense after the infection can be delimiting fungal growth by forming cell appositions to stop the disease progression. It was demonstrated that to *C. quercuum* triggers necrosis in resistant loblolly pine and forms wall appositions that are partially composed of callose to prevent infection (Gray and Amerson 1983). It was also reported that a distinct lesion formation is a type of resistance which limits *F. circinatum* growth in loblolly pine (Barrows-Broaddus and Dwinell 1983). I propose *C. quercuum* and *F. circinatum* have very different life cycles and infection styles as a result the way host responds to them should be distinct, too. Later in

the study I showed that in loblolly pine resistance to fusiform rust is not correlated to resistance /susceptibility to pitch canker and vice versa.

I also investigated *Fr1* marker co-segregation with the disease phenotype in two families that were a result of a cross between *Fr1/fr1* and *fr1/fr1* parents. The offspring from the crosses were genotyped for *Fr1* locus. I showed that *Fr1* marker information is predictive of resistance in the both greenhouse and the field as long as the inoculum is *Avr* to *Fr1* locus. Using clonally propagated material I increased the precision and also identified the ramets that escape. I showed that escape is a heritable trait in the greenhouse whereas it becomes inheritable in the field due to inoculum source that is presented for longer periods of time compared to a few minutes in the artificial inoculations.

In the last part of this study I switched gears to investigate gene expression in resistant (*Fr1/fr1*) and susceptible (*fr1/fr1*) individuals that were inoculated with water or *C. quercuum* in a time frame of 112 days. I showed that there are genes that have unique expression profiles across control vs. inoculated, resistant vs. susceptible and healthy vs. diseased. These results would be helpful to parse the disease development in susceptible loblolly pine seedlings.

Neither fusiform rust nor pitch canker disease resistance is far from being completely understood. Although this study brings us closer to the answers scientists are seeking, more research on biology of these fungal diseases is needed.

APPENDIX A
SAS SCRIPTS FOR MICROARRAY ANALYSIS

/**Fr12* is the dataset that contains treatment genotype time_point gene_name log2 expression here I am dropping all the control genes since they are causing extra noise*/

data *Fr12*; set tmp1.*Fr11*;

if gene='Blank water control' or gene='Blank' or gene='BLANK' or gene='Water Control' or gene=' - Control BAR' or gene=' - Control GFP' or gene=' - Control Globulin' or gene='SP3' or gene='SP2' or gene='SP1' or gene='Sp3' or gene='Sp1' or gene='Sp2' or gene='Spike Control Sp1' or gene='Spike Control Sp2' or gene='Spike Control Sp3' or gene='Spike Control Sp4' then delete; run;

/*after control_genes are deleted, run the array-level analysis to get rid of major effects like dye*/

proc sort data=*Fr12*; by dye gene time; run;

proc mixed data=*Fr12*; class array dye genotype treat time;

model log2i= dye genotype treat time dye*time treat*time/OutPred=no_contr;

random array; run;

/* after this delete time points=all, 6 hours, 90 mins*/

proc sort data=tmp1.Frno_contr; by genotype time; run;

data Frno_contr_r_i_6_90; set tmp1.Frno_contr;

if genotype='r' and treat='I' then delete;

if time='all' or time='6' or time='90' then delete; run;

/*now I can run the second level analysis where gene by gene*/

```
proc sort data=Frno_contr_r_i_6_90; by gene;
proc mixed; by gene; class array treat time2 dye spot_number genotype;
model resid=time2 treat genotype time2*treat treat*genotype time2*genotype
time2*genotype*treat;
random array spot_number(array); ods output Tests3=pvalno_c_r_i_6_90; run;
proc sort data=pvalno_c_r_i_6_90; by probf; run; data Gene_no_c_r_i_6_90;
set pvalno_c_r_i_6_90; proc sort; by ProbF;
where ProbF<3.85683E-07 ; run;
proc sort data=Gene_no_c_r_i_6_90; by gene; run;
/* get the significant genes , only one copy of gene should be in the list*/
data once_sig_gene_no_690ri; set Gene_no_c_r_i_6_90;
by gene; if first.gene; run;
/*here I merge the significant gene list with the residuals from the array level
analysis to get lsmeans and stdev associated with them*/
proc sort data=Frno_contr_r_i_6_90; by gene;run;
proc sort data=once_sig_gene_no_690ri; by gene;run; data res_no_690ri;
merge  Frno_contr_r_i_6_90 once_sig_gene_no_690ri; by gene; run;
proc sort data=res_no_690ri; by ProbF;
data clean_res_no_690ri; set res_no_690ri; if ProbF<0 then delete; run;
proc sort data=clean_res_no_690ri; by gene; run;
/*it is to get lsmeans and stdev*/
proc mixed; by gene; class array treat time2 dye spot_number genotype;
model resid=time2 treat genotype treat*time2*genotype;
```

```
random array spot_number(array);    lsmeans treat*time2*genotype;
 ods output LSMeans=wo_all_G_T_T_means diffs=wo_all_pdiff; run;
proc sort data=Wo_all_pdiff; by time2 _time2;
data clean_Wo_all_pdiff; set Wo_all_pdiff;
if time2=1 and _time2=1  then delete; if time2=2 and _time2=2  then delete;
if time2=3 and _time2=3  then delete;  if time2=4 and _time2=4  then delete;
if time2=5 and _time2=5  then delete;  if time2=6 and _time2=6  then delete;
if time2=1 and _time2=3  then delete;   if time2=1 and _time2=4  then delete;
if time2=1 and _time2=5  then delete; if time2=1 and _time2=6  then delete;
if time2=2 and _time2=4  then delete; if time2=2 and _time2=5  then delete;
if time2=2 and _time2=6  then delete;  if time2=3 and _time2=5  then delete;
if time2=3 and _time2=6  then delete;  if time2=4 and _time2=6  then delete;
if time2=2 and _time2=1  then delete; if time2=3 and _time2=1  then delete;
if time2=4 and _time2=1  then delete; if time2=5 and _time2=1  then delete;
if time2=6 and _time2=1  then delete; if time2=3 and _time2=2  then delete;
if time2=4 and _time2=2  then delete; if time2=5 and _time2=2  then delete;
if time2=6 and _time2=2  then delete; if time2=4 and _time2=3  then delete;
if time2=5 and _time2=3  then delete; if time2=6 and _time2=3  then delete;
if time2=5 and _time2=4  then delete;   if time2=6 and _time2=4  then delete;
if time2=6 and _time2=5  then delete; if treat='C' and _treat='I' then delete;
if genotype='R' and _genotype='r' then delete; if treat='I' and _treat='C' then delete;
if genotype='r' and _genotype='R' then delete; run;
```

APPENDIX B
ASREML SCRIPT FOR ASYMTOTIC Z-TEST

```
Test E fusiform rust only susceptible clones

 clone 440 !A

 family 61 !A

 female 32 !P

 male 32 !P

 rep 4 !A

 inc 110 !A

 row 55 !A

 col 20 !A

 ncol 40 !A

 nrow 110 !A

 score

/gck/parped.txt !ALPHA

/gck/VINF.prn

score ~ mu !r rep female and(male) family clone
```

APPENDIX C
HEALTHY VS. DISEASED GENE LIST

CloneID	Genotype	Treatment	First time increment	Second time increment	Third time increment
J4	R	C	0	0	0
J4	R	I	0	0	0
J4	S	C	0	0	0
J4	S	I	0	-1	0
07 E10	R	C	-1	0	0
07 E10	R	I	-1	0	0
07 E10	S	C	-1	0	0
07 E10	S	I	-1	0	1
37 G12	R	C	-1	0	1
37 G12	R	I	-1	0	1
37 G12	S	C	-1	0	1
37 G12	S	I	0	0	1
G1	R	C	0	0	0
G1	R	I	0	0	0
G1	S	C	0	0	0
G1	S	I	0	-1	0
G12	R	C	0	0	0
G12	R	I	0	0	0
G12	S	C	0	0	0
G12	S	I	0	-1	0
G16	R	C	0	0	0
G16	R	I	0	0	0
G16	S	C	0	0	0
G16	S	I	1	-1	-1
G17	R	C	0	0	0
G17	R	I	0	0	0
G17	S	C	0	0	0
G17	S	I	0	-1	0
G27	R	C	0	0	0
G27	R	I	0	0	0
G27	S	C	0	0	0
G27	S	I	0	-1	0
G30	R	C	0	0	0
G30	R	I	0	0	0

G30	S	C	0	0	0
G30	S	I	0	-1	0
G34	R	C	0	0	0
G34	R	I	0	0	0
G34	S	C	0	0	0
G34	S	I	0	-1	0
G35	R	C	0	0	0
G35	R	I	0	0	0
G35	S	C	0	0	0
G35	S	I	0	-1	0
G39	R	C	0	0	0
G39	R	I	0	0	0
G39	S	C	0	0	0
G39	S	I	0	-1	0
G41	R	C	0	0	0
G41	R	I	0	0	0
G41	S	C	0	0	0
G41	S	I	1	-1	0
G45	R	C	0	0	0
G45	R	I	0	0	0
G45	S	C	0	0	0
G45	S	I	1	-1	-1
G51	R	C	0	0	0
G51	R	I	0	0	0
G51	S	C	0	0	0
G51	S	I	0	-1	0
G56	R	C	0	0	0
G56	R	I	0	0	0
G56	S	C	0	0	0
G56	S	I	0	-1	0
G8	R	C	0	0	0
G8	R	I	0	0	0
G8	S	C	0	0	0
G8	S	I	0	-1	-1
H8	R	C	0	-1	0
H8	R	I	0	-1	0
H8	S	C	0	-1	0
H8	S	I	0	0	0
NXCI_002_E02	R	C	0	-1	0
NXCI_002_E02	R	I	0	-1	0
NXCI_002_E02	S	C	0	-1	0
NXCI_002_E02	S	I	0	0	0
NXCI_004_G05	R	C	-1	0	0
NXCI_004_G05	R	I	-1	0	0

NXCI_004_G05	S	C	-1	0	0
NXCI_004_G05	S	I	-1	-1	0
NXCI_018_A08	R	C	-1	0	0
NXCI_018_A08	R	I	-1	0	0
NXCI_018_A08	S	C	-1	0	0
NXCI_018_A08	S	I	-1	-1	0
NXCI_027_G06	R	C	-1	1	0
NXCI_027_G06	R	I	-1	1	0
NXCI_027_G06	S	C	-1	1	0
NXCI_027_G06	S	I	-1	0	0
NXCI_034_F04	R	C	0	0	0
NXCI_034_F04	R	I	0	0	0
NXCI_034_F04	S	C	0	0	0
NXCI_034_F04	S	I	0	-1	-1
NXCI_042_D04_F	R	C	-1	0	0
NXCI_042_D04_F	R	I	-1	0	0
NXCI_042_D04_F	S	C	-1	0	0
NXCI_042_D04_F	S	I	-1	-1	0
NXCI_057_B05	R	C	-1	0	0
NXCI_057_B05	R	I	-1	0	0
NXCI_057_B05	S	C	-1	0	0
NXCI_057_B05	S	I	0	-1	0
NXCI_067_H06	R	C	-1	0	0
NXCI_067_H06	R	I	-1	0	0
NXCI_067_H06	S	C	-1	0	0
NXCI_067_H06	S	I	-1	-1	0
NXCI_070_D01	R	C	-1	0	0
NXCI_070_D01	R	I	-1	0	0
NXCI_070_D01	S	C	-1	0	0
NXCI_070_D01	S	I	-1	-1	0
NXCI_075_D09	R	C	0	0	0
NXCI_075_D09	R	I	0	0	0
NXCI_075_D09	S	C	0	0	0
NXCI_075_D09	S	I	0	-1	0
NXCI_075_E11	R	C	-1	-1	1
NXCI_075_E11	R	I	-1	-1	1
NXCI_075_E11	S	C	-1	-1	1
NXCI_075_E11	S	I	-1	-1	0
NXCI_082_E07_F	R	C	0	-1	1
NXCI_082_E07_F	R	I	0	-1	1
NXCI_082_E07_F	S	C	0	-1	1
NXCI_082_E07_F	S	I	0	-1	0
NXCI_082_G01	R	C	-1	0	0
NXCI_082_G01	R	I	-1	0	0

NXCI_082_G01	S	C	-1	0	0
NXCI_082_G01	S	I	0	0	0
NXCI_099_A12	R	C	-1	0	0
NXCI_099_A12	R	I	-1	0	0
NXCI_099_A12	S	C	-1	0	0
NXCI_099_A12	S	I	0	0	0
NXCI_111_C10	R	C	-1	0	0
NXCI_111_C10	R	I	-1	0	0
NXCI_111_C10	S	C	-1	0	0
NXCI_111_C10	S	I	0	-1	0
NXCI_150_F06_F	R	C	-1	1	-1
NXCI_150_F06_F	R	I	-1	1	-1
NXCI_150_F06_F	S	C	-1	1	-1
NXCI_150_F06_F	S	I	-1	0	0
NXCI_153_A02_F	R	C	-1	-1	0
NXCI_153_A02_F	R	I	-1	-1	0
NXCI_153_A02_F	S	C	-1	-1	0
NXCI_153_A02_F	S	I	0	-1	0
NXCI_164_A06_F	R	C	-1	0	0
NXCI_164_A06_F	R	I	-1	0	0
NXCI_164_A06_F	S	C	-1	0	0
NXCI_164_A06_F	S	I	0	-1	0
NXLV100_F02_F	R	C	-1	0	0
NXLV100_F02_F	R	I	-1	0	0
NXLV100_F02_F	S	C	-1	0	0
NXLV100_F02_F	S	I	0	0	0
NXLV103_E01_F	R	C	-1	1	0
NXLV103_E01_F	R	I	-1	1	0
NXLV103_E01_F	S	C	-1	1	0
NXLV103_E01_F	S	I	-1	0	-1
NXLV105_B02_F	R	C	-1	0	0
NXLV105_B02_F	R	I	-1	0	0
NXLV105_B02_F	S	C	-1	0	0
NXLV105_B02_F	S	I	-1	1	0
NXLV129_C12_F	R	C	-1	0	0
NXLV129_C12_F	R	I	-1	0	0
NXLV129_C12_F	S	C	-1	0	0
NXLV129_C12_F	S	I	-1	-1	0
NXLV_012_A05_F	R	C	0	-1	0
NXLV_012_A05_F	R	I	0	-1	0
NXLV_012_A05_F	S	C	0	-1	0
NXLV_012_A05_F	S	I	0	0	0
NXLV_022_H08_F	R	C	-1	0	0
NXLV_022_H08_F	R	I	-1	0	0

NXLV_022_H08_F	S	C	-1	0	0
NXLV_022_H08_F	S	I	0	0	-1
NXLV_023_D12_F	R	C	-1	-1	0
NXLV_023_D12_F	R	I	-1	-1	0
NXLV_023_D12_F	S	C	-1	-1	0
NXLV_023_D12_F	S	I	0	0	0
NXLV_049_G11_F	R	C	-1	0	-1
NXLV_049_G11_F	R	I	-1	0	-1
NXLV_049_G11_F	S	C	-1	0	-1
NXLV_049_G11_F	S	I	-1	0	0
NXLV_079_G07_F	R	C	-1	0	0
NXLV_079_G07_F	R	I	-1	0	0
NXLV_079_G07_F	S	C	-1	0	0
NXLV_079_G07_F	S	I	-1	1	0
NXNV 129 F06	R	C	1	0	0
NXNV 129 F06	R	I	1	0	0
NXNV 129 F06	S	C	1	0	0
NXNV 129 F06	S	I	1	0	1
NXNV015H07	R	C	-1	1	-1
NXNV015H07	R	I	-1	1	-1
NXNV015H07	S	C	-1	1	-1
NXNV015H07	S	I	-1	1	0
NXNV027F10	R	C	0	1	0
NXNV027F10	R	I	0	1	0
NXNV027F10	S	C	0	1	0
NXNV027F10	S	I	0	0	0
NXNV_073_G04	R	C	-1	1	0
NXNV_073_G04	R	I	-1	1	0
NXNV_073_G04	S	C	-1	1	0
NXNV_073_G04	S	I	0	0	0
NXNV_096_C09	R	C	0	0	0
NXNV_096_C09	R	I	0	0	0
NXNV_096_C09	S	C	0	0	0
NXNV_096_C09	S	I	0	0	-1
NXNV_118_E06	R	C	-1	0	0
NXNV_118_E06	R	I	-1	0	0
NXNV_118_E06	S	C	-1	0	0
NXNV_118_E06	S	I	0	-1	0
NXNV_122_C07_F	R	C	-1	0	0
NXNV_122_C07_F	R	I	-1	0	0
NXNV_122_C07_F	S	C	-1	0	0
NXNV_122_C07_F	S	I	0	0	0
NXNV_125_E12_F	R	C	-1	0	0
NXNV_125_E12_F	R	I	-1	0	0

NXNV_125_E12_F	S	C	-1	0	0
NXNV_125_E12_F	S	I	-1	-1	0
NXNV_132_G11_F	R	C	-1	1	0
NXNV_132_G11_F	R	I	-1	1	0
NXNV_132_G11_F	S	C	-1	1	0
NXNV_132_G11_F	S	I	-1	0	0
NXNV_135_E01_F	R	C	-1	-1	0
NXNV_135_E01_F	R	I	-1	-1	0
NXNV_135_E01_F	S	C	-1	-1	0
NXNV_135_E01_F	S	I	0	-1	0
NXNV_147_G03_F	R	C	-1	1	0
NXNV_147_G03_F	R	I	-1	1	0
NXNV_147_G03_F	S	C	-1	1	0
NXNV_147_G03_F	S	I	0	0	0
NXNV_159_G03	R	C	0	-1	0
NXNV_159_G03	R	I	0	-1	0
NXNV_159_G03	S	C	0	-1	0
NXNV_159_G03	S	I	0	0	0
NXNV_163_F07_F	R	C	-1	0	0
NXNV_163_F07_F	R	I	-1	0	0
NXNV_163_F07_F	S	C	-1	0	0
NXNV_163_F07_F	S	I	0	0	0
NXNV_173_B11_F	R	C	-1	1	0
NXNV_173_B11_F	R	I	-1	1	0
NXNV_173_B11_F	S	C	-1	1	0
NXNV_173_B11_F	S	I	0	0	0
NXPV_062_E04_F	R	C	-1	0	0
NXPV_062_E04_F	R	I	-1	0	0
NXPV_062_E04_F	S	C	-1	0	0
NXPV_062_E04_F	S	I	0	0	0
NXPV_068_E06_F	R	C	-1	1	-1
NXPV_068_E06_F	R	I	-1	1	-1
NXPV_068_E06_F	S	C	-1	1	-1
NXPV_068_E06_F	S	I	-1	1	0
NXPV_076_C12_F	R	C	-1	1	-1
NXPV_076_C12_F	R	I	-1	1	-1
NXPV_076_C12_F	S	C	-1	1	-1
NXPV_076_C12_F	S	I	-1	1	0
NXRV064_C07_F	R	C	-1	0	-1
NXRV064_C07_F	R	I	-1	0	-1
NXRV064_C07_F	S	C	-1	0	-1
NXRV064_C07_F	S	I	-1	0	0
NXRV079_D01_F	R	C	-1	1	-1
NXRV079_D01_F	R	I	-1	1	-1

NXRV079_D01_F	S	C	-1	1	-1
NXRV079_D01_F	S	I	-1	1	0
NXRV118_B08_F	R	C	-1	0	0
NXRV118_B08_F	R	I	-1	0	0
NXRV118_B08_F	S	C	-1	0	0
NXRV118_B08_F	S	I	-1	-1	0
NXSI 008 G11	R	C	0	-1	0
NXSI 008 G11	R	I	0	-1	0
NXSI 008 G11	S	C	0	-1	0
NXSI 008 G11	S	I	1	-1	-1
NXSI 059 G09	R	C	0	-1	0
NXSI 059 G09	R	I	0	-1	0
NXSI 059 G09	S	C	0	-1	0
NXSI 059 G09	S	I	1	-1	0
NXSI 102 F11	R	C	-1	-1	0
NXSI 102 F11	R	I	-1	-1	0
NXSI 102 F11	S	C	-1	-1	0
NXSI 102 F11	S	I	0	-1	0
NXSI_013_C04	R	C	0	-1	0
NXSI_013_C04	R	I	0	-1	0
NXSI_013_C04	S	C	0	-1	0
NXSI_013_C04	S	I	1	-1	0
NXSI_027_G10	R	C	-1	0	0
NXSI_027_G10	R	I	-1	0	0
NXSI_027_G10	S	C	-1	0	0
NXSI_027_G10	S	I	-1	-1	0
NXSI_040_C01	R	C	-1	0	0
NXSI_040_C01	R	I	-1	0	0
NXSI_040_C01	S	C	-1	0	0
NXSI_040_C01	S	I	-1	-1	0
NXSI_041_B01	R	C	0	-1	0
NXSI_041_B01	R	I	0	-1	0
NXSI_041_B01	S	C	0	-1	0
NXSI_041_B01	S	I	0	0	0
NXSI_055_H08	R	C	-1	0	0
NXSI_055_H08	R	I	-1	0	0
NXSI_055_H08	S	C	-1	0	0
NXSI_055_H08	S	I	-1	1	0
NXSI_060_E02	R	C	-1	0	0
NXSI_060_E02	R	I	-1	0	0
NXSI_060_E02	S	C	-1	0	0
NXSI_060_E02	S	I	0	-1	0
NXSI_063_D01	R	C	-1	1	0
NXSI_063_D01	R	I	-1	1	0

NXSI_063_D01	S	C	-1	1	0
NXSI_063_D01	S	I	-1	0	0
NXSI_064_A03	R	C	1	-1	0
NXSI_064_A03	R	I	1	-1	0
NXSI_064_A03	S	C	1	-1	0
NXSI_064_A03	S	I	1	-1	-1
NXSI_067_F10_F	R	C	-1	0	0
NXSI_067_F10_F	R	I	-1	0	0
NXSI_067_F10_F	S	C	-1	0	0
NXSI_067_F10_F	S	I	0	0	0
NXSI_069_F12_F	R	C	-1	1	-1
NXSI_069_F12_F	R	I	-1	1	-1
NXSI_069_F12_F	S	C	-1	1	-1
NXSI_069_F12_F	S	I	-1	0	0
NXSI_076_E08	R	C	-1	1	0
NXSI_076_E08	R	I	-1	1	0
NXSI_076_E08	S	C	-1	1	0
NXSI_076_E08	S	I	-1	0	0
NXSI_092_E10	R	C	-1	-1	1
NXSI_092_E10	R	I	-1	-1	1
NXSI_092_E10	S	C	-1	-1	1
NXSI_092_E10	S	I	-1	-1	0
NXSI_098_C01	R	C	1	-1	0
NXSI_098_C01	R	I	1	-1	0
NXSI_098_C01	S	C	1	-1	0
NXSI_098_C01	S	I	1	-1	-1
NXSI_099_H06_F	R	C	-1	0	0
NXSI_099_H06_F	R	I	-1	0	0
NXSI_099_H06_F	S	C	-1	0	0
NXSI_099_H06_F	S	I	-1	1	0
NXSI_101_B01	R	C	-1	0	0
NXSI_101_B01	R	I	-1	0	0
NXSI_101_B01	S	C	-1	0	0
NXSI_101_B01	S	I	-1	-1	0
NXSI_103_D11_F	R	C	-1	1	0
NXSI_103_D11_F	R	I	-1	1	0
NXSI_103_D11_F	S	C	-1	1	0
NXSI_103_D11_F	S	I	-1	0	0
NXSI_103_E12_F	R	C	-1	1	-1
NXSI_103_E12_F	R	I	-1	1	-1
NXSI_103_E12_F	S	C	-1	1	-1
NXSI_103_E12_F	S	I	-1	1	0
NXSI_104_B11	R	C	0	0	1
NXSI_104_B11	R	I	0	0	1

NXSI_104_B11	S	C	0	0	1
NXSI_104_B11	S	I	0	1	1
NXSI_114_A04	R	C	0	-1	1
NXSI_114_A04	R	I	0	-1	1
NXSI_114_A04	S	C	0	-1	1
NXSI_114_A04	S	I	0	-1	0
NXSI_115_A12_F	R	C	-1	0	0
NXSI_115_A12_F	R	I	-1	0	0
NXSI_115_A12_F	S	C	-1	0	0
NXSI_115_A12_F	S	I	0	0	0
NXSI_133_G11	R	C	0	-1	0
NXSI_133_G11	R	I	0	-1	0
NXSI_133_G11	S	C	0	-1	0
NXSI_133_G11	S	I	0	0	0
pi134-1	R	C	0	1	0
pi134-1	R	I	0	1	0
pi134-1	S	C	0	1	0
pi134-1	S	I	0	0	0
pi150-2	R	C	0	-1	1
pi150-2	R	I	0	-1	1
pi150-2	S	C	0	-1	1
pi150-2	S	I	0	-1	0
pi226	R	C	0	0	0
pi226	R	I	0	0	0
pi226	S	C	0	0	0
pi226	S	I	0	-1	0
pi243	R	C	0	0	0
pi243	R	I	0	0	0
pi243	S	C	0	0	0
pi243	S	I	1	-1	-1
pi266	R	C	0	-1	1
pi266	R	I	0	-1	1
pi266	S	C	0	-1	1
pi266	S	I	0	0	1
pi295	R	C	0	0	0
pi295	R	I	0	0	0
pi295	S	C	0	0	0
pi295	S	I	1	0	-1
pi310	R	C	1	0	0
pi310	R	I	1	0	0
pi310	S	C	1	0	0
pi310	S	I	1	-1	0
pi64-9	R	C	0	0	0
pi64-9	R	I	0	0	0

pi64-9	S	C	0	0	0
pi64-9	S	I	0	-1	-1

APPENDIX D
GENES THAT ARE REGULATED ACROSS TIME

01 D11	01 F09
01 F11	02 A06
02 B01	02 B03
02 C04	02 D01
02 G09	03 B07
03 D10	03 E05
03 F07	03 G03
03 H06	04 A02
04 D09	04 E10
04 F05	04 H02
06 A10	06 B06
06 C03	06 C04
06 F05	06 G07
06 H04	06 H05
07 D02	07 E10
08 A10	08 B05
08 H06	12 D06
12 E03	12 E05
13 C12	13 F12
13 H06	14 G06

15 C08	15 G05
16 C01	21 B10
21 E01	21 E10
22 H10	23 A08
23 E11	23 F03
23 G12	24 F06
25 E06	27 B08
27 G04	27 G09
27 G10	30 C04
30 G05	30 H08
33 H11	34 E09
37 D08	37 D12
37 E04	37 E10
37 F05	37 G12
38 F06	38 G04
40 A03	40 D05
40 E09	40 F04
Eli3	G1
G10	G11
G12	G13
G16	G17
G18	G19
G2	G26

G27	G3
G30	G31
G32	G33
G34	G35
G36	G37
G38	G39
G41	G45
G46	G51
G55	G56
G57	
G59	G6
G60	G63
G65	G66
G67	G68
G7	G72
G73	G75
G8	G9
H16	H17
H21	H24
H25	H27
H39	H5
H6	H7
H8	J10

J11	J16
J18	J19
J2	J4
NXCI 048 F02	NXCI 056 A03
NXCI 069 B02	NXCI 147 C04
NXCI_002_A12	NXCI_002_D01_F
NXCI_002_E02	NXCI_002_E07
NXCI_002_G10_F	NXCI_002_G11_F
NXCI_002_H01_F	NXCI_002_H03_F
NXCI_002_H04	NXCI_004_G05
NXCI_005_G03	NXCI_007_G08_F
NXCI_008_C01	NXCI_008_D06_F
NXCI_008_D12_F	NXCI_008_F09_F
NXCI_008_G03_F	NXCI_008_H07_F
NXCI_008_H10	NXCI_009_A10
NXCI_009_B05_F	NXCI_009_B08_F
NXCI_018_A08	NXCI_018_D03
NXCI_018_H04	NXCI_019_E11
NXCI_020_A02	NXCI_020_G08_F
NXCI_021_D03	NXCI_022_E07
NXCI_022_G01_F	NXCI_023_D01
NXCI_023_F12	NXCI_025_F02
NXCI_025_G06	NXCI_026_C06

NXCI_026_D09_F	NXCI_026_F10
NXCI_027_E04	NXCI_027_F06_F
NXCI_027_F08_F	NXCI_027_G06
NXCI_028_B02_F	NXCI_029_G10_F
NXCI_029_H10_F	NXCI_031_A08_F
NXCI_031_C04_F	NXCI_031_E05
NXCI_033_C02	NXCI_033_F03
NXCI_034_B01	NXCI_034_F04
NXCI_037_B03_F	NXCI_040_B11
NXCI_041_E04	NXCI_042_D04_F
NXCI_042_D08	NXCI_043_A11
NXCI_043_F09_F	NXCI_044_A12
NXCI_044_F11_F	NXCI_045_C01
NXCI_045_G05_F	NXCI_046_E05
NXCI_047_A08_F	NXCI_047_C05
NXCI_048_H04	NXCI_050_F08
NXCI_055_C01	NXCI_055_C06
NXCI_057_B05	NXCI_057_E03
NXCI_057_E05	NXCI_058_C02
NXCI_058_H01_F	NXCI_060_A12_F
NXCI_061_B09	NXCI_061_F02_F
NXCI_062_H01_F	NXCI_064_E04
NXCI_066_A11	NXCI_066_F01

NXCI_066_G08	NXCI_066_H04
NXCI_067_A10	NXCI_067_H06
NXCI_069_A02	NXCI_069_H11
NXCI_070_B10	NXCI_070_D01
NXCI_070_E11	NXCI_070_G08
NXCI_071_B03	NXCI_071_C01
NXCI_071_F03	NXCI_075_B02_F
NXCI_075_C07	NXCI_075_D09
NXCI_075_E11	NXCI_076_A09
NXCI_076_A10	NXCI_076_E05_F
NXCI_076_F07	NXCI_076_F09
NXCI_076_G08_F	NXCI_082_E07
NXCI_082_E07_F	NXCI_082_G01
NXCI_082_G01_F	NXCI_083_A06
NXCI_083_D09_F	NXCI_084_A07_F
NXCI_084_G02	NXCI_085_E04
NXCI_085_E12	NXCI_085_H12
NXCI_086_A09	NXCI_087_F06
NXCI_087_F07	NXCI_093_E01
NXCI_093_H05	NXCI_094_C09
NXCI_094_C11	NXCI_094_E12
NXCI_094_G11	NXCI_095_C01
NXCI_095_D10	NXCI_096_A07

NXCI_096_C05	NXCI_097_A07
NXCI_098_D10	NXCI_098_F10
NXCI_099_A12	NXCI_101_B10
NXCI_102_F06	NXCI_103_A12
NXCI_106_C10	NXCI_106_F03_F
NXCI_107_G09_F	NXCI_107_H04_F
NXCI_108_E05	NXCI_109_F09
NXCI_111_C10	NXCI_114_B08
NXCI_115_C04	NXCI_116_C11_F
NXCI_116_D01	NXCI_118_F05
NXCI_122_H05	NXCI_124_C07
NXCI_125_D10_F	NXCI_127_D04_F
NXCI_128_G07_F	NXCI_130_C09
NXCI_131_H09	NXCI_132_B08_F
NXCI_132_B11	NXCI_132_E06
NXCI_132_G02_F	NXCI_132_H04
NXCI_134_B04_F	NXCI_134_H12
NXCI_136_A08	NXCI_144_F06
NXCI_149_E03	NXCI_149_F01
NXCI_149_F01_F	NXCI_149_H12
NXCI_150_A07	NXCI_150_F06_F
NXCI_151_G03_F	NXCI_153_A02_F
NXCI_153_F03_F	NXCI_153_G06

NXCI_153_G06_F	NXCI_153_H08_F
NXCI_155_E05_F	NXCI_155_H03
NXCI_155_H06_F	NXCI_157_D11_F
NXCI_162_A05_F	NXCI_164_A06_F
NXCI_164_B04_F	NXCI_164_C11_F
NXLV082_A10_F	NXLV082_F03_F
NXLV085_B05_F	NXLV088_B11_F
NXLV090_G07_F	NXLV098_B10_F
NXLV098_E06_F	NXLV100_B02_F
NXLV100_F02_F	NXLV101_A05_F
NXLV101_G02_F	NXLV103_E01_F
NXLV105_B02_F	NXLV105_E07_F
NXLV106_G06_F	NXLV106_G10_F
NXLV109_B02_F	NXLV111_E05_F
NXLV112_B02_F	NXLV112_H10_F
NXLV118_B08_F	NXLV127_E02_F
NXLV129_C12_F	NXLV133_D07_F
NXLV_007_D11_F	NXLV_009_A11_F
NXLV_010_H05_F	NXLV_012_A05_F
NXLV_014_C04_F	NXLV_020_A08_F
NXLV_020_E04_F	NXLV_022_E02_F
NXLV_022_H08_F	NXLV_023_D12_F
NXLV_024_A02_F	NXLV_024_G06_F

NXLV_029_D05_F

NXLV_030_A06_F

NXLV_031_D02_F

NXLV_037_E01_F

NXLV_039_H10_F

NXLV_041_H10_F

NXLV_049_G11_F

NXLV_065_A09_F

NXLV_070_F01_F

NXLV_077_B11_F

NXLV_077_H10_F

NXLV_079_G07_F

NXLV_080_H03_F

NXNV 074 G09

NXNV 100 H01

NXNV 118 C02

NXNV 129 F06

NXNV002C02

NXNV003F06

NXNV005A03

NXNV005D01

NXNV005H04

NXNV006A11

NXNV015F05

NXNV015H07

NXNV018A10

NXNV019E06

NXNV021C03

NXNV027B07

NXNV027F10

NXNV028A02

NXNV047B02

NXNV047B05

NXNV047E10

NXNV_001_B08

NXNV_005_B04

NXNV_007_G05

NXNV_007_G06

NXNV_008_F05

NXNV_009_C04

NXNV_010_H01

NXNV_015_H07

NXNV_018_E08

NXNV_044_C04

NXNV_044_D09

NXNV_044_G02

NXNV_044_G05	NXNV_045_E12
NXNV_046_A05	NXNV_046_B04
NXNV_046_D01	NXNV_046_F03
NXNV_048_F06	NXNV_060_F07
NXNV_060_H10	NXNV_066_B07
NXNV_067_A11	NXNV_067_B01
NXNV_070_F06	NXNV_071_H03
NXNV_072_F02_F	NXNV_072_G08_F
NXNV_073_G04	NXNV_074_G01
NXNV_074_G06	NXNV_074_H01_F
NXNV_078_B01_F	NXNV_079_G02
NXNV_081_D10	NXNV_083_A10
NXNV_083_D09	NXNV_083_E04
NXNV_083_E11_F	NXNV_086_B04
NXNV_086_C07	NXNV_089_A02
NXNV_089_B08	NXNV_089_B08_F
NXNV_089_C07_F	NXNV_089_E08_F
NXNV_089_G04	NXNV_091_F02
NXNV_095_B07_F	NXNV_095_C08_F
NXNV_095_F08_F	NXNV_096_A01_F
NXNV_096_A04	NXNV_096_C09
NXNV_096_E01	NXNV_098_D05
NXNV_105_D01_F	NXNV_106_A05

NXNV_106_A11_F	NXNV_106_F12_F
NXNV_108_E11_F	NXNV_108_G08_F
NXNV_117_G05_F	NXNV_118_E06
NXNV_120_E10	NXNV_120_F03_F
NXNV_120_F05	NXNV_120_G04_F
NXNV_120_H02	NXNV_122_C07_F
NXNV_122_E06_F	NXNV_123_B06
NXNV_124_H02_F	NXNV_125_E12_F
NXNV_127_E04	NXNV_127_F09
NXNV_128_D06_F	NXNV_129_G07
NXNV_130_G07	NXNV_131_F05
NXNV_131_F08	NXNV_132_B03
NXNV_132_G06_F	NXNV_132_G11_F
NXNV_133_A09_F	NXNV_133_G06
NXNV_133_H03_F	NXNV_134_H10
NXNV_135_E01_F	NXNV_136_C05_F
NXNV_136_C12_F	NXNV_139_B12
NXNV_139_C09	NXNV_139_C11_F
NXNV_144_C01_F	NXNV_145_F12
NXNV_147_G03_F	NXNV_147_H08
NXNV_148_E12_F	NXNV_148_G06_F
NXNV_149_B07	NXNV_149_E07
NXNV_150_D05_F	NXNV_151_G10

NXNV_153_F09

NXNV_158_B06_F

NXNV_158_D09

NXNV_159_C03_F

NXNV_159_G03

NXNV_160_C09

NXNV_160_C10

NXNV_162_D12_F

NXNV_162_H07

NXNV_162_H07_F

NXNV_163_F07_F

NXNV_163_G07

NXNV_163_G09

NXNV_163_G10_F

NXNV_164_H08

NXNV_173_B11_F

NXNV_173_C05_F

NXNV_173_E07

NXNV_181_A11_F

NXNV_181_B11_F

NXNV_181_F08_F

NXNV_181_H08

NXNV_185_D06

NXNV_185_F02

NXNV_186_A10

NXNV_186_C11_F

NXNV_186_G05

NXPV_007_H07_F

NXPV_008_H09_F

NXPV_010_A07_F

NXPV_010_B09_F

NXPV_010_C08

NXPV_010_E07_F

NXPV_010_H01_F

NXPV_011_F10_F

NXPV_012_H12_F

NXPV_013_A04_F

NXPV_013_C08_F

NXPV_020_G09_F

NXPV_021_F10_F

NXPV_021_G10_F

NXPV_025_E07_F

NXPV_028_D12_F

NXPV_028_H06_F

NXPV_035_A07_F

NXPV_037_C02_F

NXPV_041_A04_F	NXPV_041_B08_F
NXPV_042_C11_F	NXPV_044_F01_F
NXPV_048_C09_F	NXPV_049_C03_F
NXPV_052_D04_F	NXPV_055_C02_F
NXPV_056_F09_F	NXPV_062_E04_F
NXPV_062_E05_F	NXPV_063_A12_F
NXPV_066_B11_F	NXPV_068_E06_F
NXPV_075_B11_F	NXPV_076_A06_F
NXPV_076_C12_F	NXPV_077_B05_F
NXPV_078_G08_F	NXPV_079_D06_F
NXPV_088_C08_F	NXPV_094_G04_F
NXPV_096_D02_F	NXPV_097_B11_F
NXPV_108_B07_F	NXPV_123_C07_F
NXPV_128_F03_F	NXPV_133_B10_F
NXRV055_C03_F	NXRV060_D09_F
NXRV060_D10_F	NXRV061_G09_F
NXRV061_H10_F	NXRV062_H08_F
NXRV064_C07_F	NXRV064_G04_F
NXRV066_G02_F	NXRV066_H10_F
NXRV072_A01_F	NXRV075_C07_F
NXRV077_A04_F	NXRV078_H08_F
NXRV079_D01_F	NXRV084_E09_F
NXRV087_B06_F	NXRV097_A07_F

NXRV100_H07_F	NXRV101_H07_F
NXRV107_G02_F	NXRV112_F01_F
NXRV112_F11_F	NXRV114_B02_F
NXRV117_A05_F	NXRV118_B08_F
NXRV118_E03_F	NXRV120_F06_F
NXRV125_B10_F	NXRV126_E10_F
NXRV128_D08_F	NXRV130_E04_F
NXRV132_G06_F	NXRV_003_A04_F
NXRV_003_H02_F	NXRV_011_E07_F
NXRV_016_F09_F	NXRV_017_A01_F
NXRV_022_D06_F	NXRV_025_D11_F
NXRV_025_E11_F	NXRV_025_E12_F
NXRV_037_H06_F	NXS1_121_C05
NXSI 044 F04	NXSI 060 H06
NXSI 124 B08	NXSI 008 G11
NXSI 028 D09	NXSI 059 G09
NXSI 102 F11	NXSI 117 B05
NXSI 123 D02	NXSI_007_A07_F
NXSI_012_H11	NXSI_013_C04
NXSI_021_B12	NXSI_021_D01
NXSI_021_E09	NXSI_025_H02
NXSI_027_G10	NXSI_028_G05
NXSI_029_F11	NXSI_030_C06

NXSI_031_G08	NXSI_036_C05
NXSI_039_D01	NXSI_040_C01
NXSI_040_D02	NXSI_040_D03_F
NXSI_041_B01	NXSI_041_H12
NXSI_042_G07_F	NXSI_042_H05
NXSI_043_C03	NXSI_043_H03
NXSI_044_C10	NXSI_045_A04
NXSI_045_B09	NXSI_045_G03
NXSI_046_B05	NXSI_047_A11_F
NXSI_049_A04_F	NXSI_050_C07_F
NXSI_051_G07	NXSI_053_B02
NXSI_053_G04	NXSI_053_G05
NXSI_054_A01	NXSI_054_A09
NXSI_055_B06	NXSI_055_F11
NXSI_055_H08	NXSI_056_F07
NXSI_058_B04	NXSI_058_G02
NXSI_060_B07	NXSI_060_E02
NXSI_061_F04_F	NXSI_062_E07
NXSI_063_D01	NXSI_063_E04
NXSI_064_A03	NXSI_064_H06
NXSI_065_C08	NXSI_067_C11
NXSI_067_F10_F	NXSI_067_H09_F
NXSI_068_G09	NXSI_069_F12_F

NXSI_073_F05	NXSI_075_B04_F
NXSI_076_E08	NXSI_077_F09
NXSI_079_D06	NXSI_079_D09_F
NXSI_081_D01	NXSI_082_H01
NXSI_083_G10_F	NXSI_088_C05_F
NXSI_089_E04	NXSI_089_H07
NXSI_090_C05	NXSI_092_E10
NXSI_092_H03_F	NXSI_096_G02_F
NXSI_097_H07	NXSI_098_A04
NXSI_098_C01	NXSI_099_F06
NXSI_099_F10	NXSI_099_G06
NXSI_099_H06_F	NXSI_100_A04
NXSI_100_C11	NXSI_100_D07
NXSI_100_F02	NXSI_100_F12
NXSI_101_B01	NXSI_101_E11
NXSI_101_H03_F	NXSI_102_D03
NXSI_102_F12	NXSI_102_H05
NXSI_103_A08	NXSI_103_A10
NXSI_103_B01	NXSI_103_C04
NXSI_103_D11_F	NXSI_103_E12_F
NXSI_103_F08	NXSI_103_H03
NXSI_104_B11	NXSI_104_E11
NXSI_104_H10	NXSI_105_G10

NXSI_107_C09

NXSI_108_D12

NXSI_108_H05

NXSI_112_B07

NXSI_112_D01

NXSI_112_D08

NXSI_112_G05_F

NXSI_113_B09

NXSI_113_C10_F

NXSI_113_D07

NXSI_113_E06_F

NXSI_113_G11_F

NXSI_113_H02

NXSI_114_A04

NXSI_114_D12

NXSI_114_G07

NXSI_116_A11

NXSI_116_B04

NXSI_116_F02

NXSI_117_B05

NXSI_117_C06_F

NXSI_118_A03

NXSI_118_B03

NXSI_118_F05_F

NXSI_119_D08

NXSI_119_F11

NXSI_120_D02_F

NXSI_121_A05

NXSI_121_F04_F

NXSI_122_H10

NXSI_124_C04

NXSI_127_E02

NXSI_131_C03

NXSI_132_F03

NXSI_132_H01

NXSI_115_A12_F

NXSI_133_B03_F

NXSI_133_B05

NXSI_133_G06

NXSI_133_G11

NXSI_134_E02

NXSI_136_C07_F

NXSI_137_D09

NXSI_137_E06

NXSI_142_F05

NXSI_143_G11

NXSI_144_H01

NXSI_145_D04

NXSI_145_E11_F

PC 03 F07

PC 04 B12

PC 05 A11

PC 07 F10

PC 18 B08

PC 19 E01

ST 32 C09

ST_02_E09

ST_06_F05

ST_15_G05

ST_19_A09

ST_20_B02

ST_21_E01

ST_22_F09

ST_23_F07

ST_24_H10

ST_25_C07

ST_29_A08

ST_35_A01

ST_35_B03

ST_35_D08

ST_36_C08

ST_37_B11

ST_40_A03

pi107-2

pi111-4

pi113-1

pi115-1

pi118-1

pi121-1

pi129-1

pi134-1

pi143-1

pi148-1

NXSI_136_H09_F

pi150-2

pi152-5

pi167-5

pi191-2

pi193-3

pi194-1

pi196-1

pi201-2

pi226

pi235

pi240

pi243

pi255

pi261

pi263

pi266

pi267

pi270

pi271

pi273

pi274

pi275

pi278

pi284

pi287

pi288

pi293

pi295

pi305

pi306

pi310

pi311b

pi315

pi46-1

pi54-5

pi59-1

pi64-9

pi70-2

pi73-1

pi76-1

pi78-1

pi79-2

pi90-2

pi97-3

pic56-12

pidd1

APPENDIX E
MICROARRAY PROCEDURE

Indirect Incorporation of Cy Dyes

SuperScript Indirect cDNA Labeling from total RNA

First-Strand cDNA Synthesis using Invitrogen's SuperScript Indirect cDNA Labeling Kit (Catalog numbers L1014-01 and L1014-02).

What follows is the protocol I use for cDNA synthesis from total RNA and indirect cDNA labeling prior to microarray hybridizations. The protocol below is similar to that in the instruction manual provided with this kit but has been modified previously by Dr. Rob Alba. This is a copy of his procedure and I have added a couple of my own modifications. His originals can be found at

http://ted.bti.cornell.edu/array/interface/protocol/protocol.html

First-Strand cDNA Synthesis Rxn.

Mix and briefly spin each kit component before use.

Prepare rxns as follows:

Xul DEPC-H2O

Xul Rnase free Dnase treated total RNA (15 to 20 ug/rxn)

2ul Anchored Oligo(dT)20 Primer (2.5ug/ul)

1ul Random Hexamer

Total Volume = 18ul

Incubate tubes at 70^{o}C for 5 min, and then place on ice for at least 1 min.

Add the following to each rxn tube on ice:

6ul 5X First-Strand buffer

1.5ul 0.1 M DTT

1.5ul 10-mM dNTP mix

1ul RNaseOUT (40U/ul)

2ul SuperScript III RT (400U/ul)

Total Volume = 30ul

Mix gently and spin briefly. Incubate tube at 46oC Overnight

Add 15ul of 1N NaOH to each rxn tube and mix thoroughly.

Incubate tube at 70o C for 10 min.

Add 15ul of 1N HCl; mix gently.

Add 20ul 3M NaOAc (pH 5.2); mix gently.

Purifying First-Strand cDNA.

Add 500ul of Loading Buffer to the cDNA (from Step 9) and mix well.

Place a SNAP Column on a collection tube and load your cDNA on the column.

Spin at 14,000g at room temp for 1 min; discard the flow-through.

Place the SNAP Column onto the same collection tube and add 500ul of Wash Buffer.

Spin at 14,000g at room temp for 1 min; discard the flow-through.

Repeat Steps 4 and 5 twice more, for a total of three 500ul washes.

Spin one more time at 14,000g at room temp for 1 min; discard the flow-through.

Place the SNAP Column onto a new 1.5-ml tube.

Add 50ul of DEPC-treated water to the SNAP Column and incubate at room temp for 1 min. Elute the cDNA via spin at 14,000g at room temp for 1 min.

Repeat Step 9, using the same 1.5-ml tube.

Add 10ul of 3M sodium acetate (pH 5.2) to the eluent from steps 9 and 10.

Add 4ul of glycogen (20mg/ml) to the tube and mix.

Add 250ul of ice-cold 100% EtOH, and incubate the tube -80oC for 30 min.

Spin the tube at 14,000g at 4oC for 20 min. Carefully remove the supernatant.

Add 500ul of ice-cold 70% EtOH and spin the tube at 14,000g for 2 min. Carefully remove the supernatant.

Air dry the sample for 5-10 min; ensure that all EtOH is removed. I can let it sit for a few weeks in fridge.

Warm the 2X Coupling Buffer at 37oC for 5 min and re-suspend the cDNA sample in 5ul of warm 2X Coupling Buffer. Heat the cDNA/Coupling buffer at 50oC for 10 min and vortex well. Ensure that your cDNA pellet is fully re-suspended in the 2X Coupling Buffer.

Labeling with Fluorescent Dye. When preparing the rxn, be careful to minimize exposure of the dye solution to light. Also, DMSO is hygroscopic and will absorb moisture from the air, which will react with the NHS ester of the dye and significantly reduce the coupling rxn efficiency. Keep the DMSO supplied in the kit in an amber screw-capped vial at -20oC, and let the vial warm to room temperature before opening to prevent condensation. Use only the DMSO provided with this kit.

Open one packet of Cy3- or Cy5- dye and add 45ul of DMSO directly to the dye vial.

Add 5ul of the DMSO/dye solution to the tube from Step 17.

Mix well and incubate the tube at room temp in the dark for 1 hr.

Add 20ul of 3M Sodium Acetate (pH 5.2) to the dye-coupled cDNA solution.

Add 500ul of Loading Buffer to the cDNA solution. Mix well by vortexing.

Place a SNAP Column onto a clear collection tube and load the cDNA/buffer solution.

Spin at 14,000g at room temp for 1 min; discard the flow-through.

Place the SNAP Column on the same collection tube; add 500ul of Wash Buffer to column.

Spin at 14,000g at room temp for 1 min; discard the flow-through.

Repeat Steps 8-9 three times, for a total of four 500ul washes.

Spin one more time at 14,000g at room temp for 1 min; discard the flow-through.

Place the SNAP Column onto a new amber collection tube.

Add 63ul of DEPC-water to the SNAP Column and incubate at room temp for 1 min.

Spin at 14,000g at room temp for 1 min and collect the flow-through. The flow-through should contain 60ul of your purified dye-coupled cDNA.

Assessing the Labeling Procedure. Use UV/VIS spectroscopy to assess the labeling procedure prior to microarray hybridization. This technique is described briefly in the Appendix of the Instruction Manual for the Invitrogen cDNA labeling kit (page 8). Additional information for assessing your labeling rxns can be obtained from the TIGR website (TIGR Standard Operating Procedure #M004). Optimal labeling reactions have the following characteristics: A260 > 0.5; A450 (for Cy3 labeling) < 0.2; A520 (for Cy5 labeling) < 0.2; pmols cDNA > 1000; pmols Cy > 100; nucleotides per Cy molecule < 50. The Instruction Manual for Corning's Pronto!Plus kit suggests that Frequency of Incorporation (FOI) and the FOI/Yield Ratio be determined as well. Preferably, FOI ≈ 20 to 50, and FOI/Yield Ratio ≈ 4 to 8.5%

Hybridization

What follows is the protocol I use for pre-hybridization, hybridization, washes, and subsequent scanning of our Pine microarrays. These protocols were derived from similar ones developed by Dr. Rob Alba at the Boyce Thompson Institute at Cornell University http://ted.bti.cornell.edu/ The pre-hybridization and wash protocols derive from the lab of John Quackenbush at TIGR (http://atarrays.tigr.org/PDF/Probehyb.pdf), with a few modifications.

Reagents/Materials Required

Pre-hybridization ("block") solution, (5X SSC, 0.1% SDS, 1% BSA)

Filter pre-hyb solution using a 0.2um Corning filter unit

Wash Solution #1 (1X SSC, 0.2% SDS; pre-heat to 43oC)

Filter wash solution using a 0.2um Corning filter unit

Wash Solution #2 (0.1X SSC, 0.2% SDS; room temperature)

Filter wash solution using a 0.2um Corning filter unit

Wash Solution #3 (0.1X SSC; room temperature)

Filter wash solution using a 0.2um Corning filter unit

Isopropyl alcohol

0.1% SDS

Milli-Q® H20

Coplin staining jars

LifterSlipsTM(Erie Scientific Co.; catalog #22X50I-2-4711)

50mL FalconTM tubes (place the cap from a 14mL FalconTM culture tube at the bottom of each 50mL FalconTM tube to elevate the arrays during centrifugation)

Genomic Solutions Hybridization Chambers (catalog # JHYB200003)

Hybridization Solution: 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS, 5mM potassium phosphate monobasic. Filter hyb solution using a 0.2um Corning filter unit.

Pre-hybridization/Blocking the Arrays

Pre-heat (to 43oC) □60mL of the filtered pre-hybridization solution in a Coplin jar.

Incubate arrays in warm pre-hybridization solution for 45min.

Rinse the "blocked" arrays via 5 dips in Milli-Q® H20, 5 more dips in fresh Milli-Q® H20, and three dips in 100% isopropyl alcohol.

Quickly dry the rinsed arrays by centrifugation (1min; 1000Â–1500 × rpm) in a 50mL FalconTM tube.

Do not let arrays dry out prior to centrifugation.

Excessive centrifugal force will crack array slides.

Inspect arrays carefully after centrifugation; if they are not clean, repeat steps 3 and 4.

Use arrays immediately after pre-hybridization.

Preparation of Cy-Labeled cDNA Targets

Conduct a spectrophotometric assay to asses the success of each labeling reaction using the procedure described in TIGR's Standard Operating Procedure #M004.

Refer to: http://pga.tigr.org/sop/M004_1a.pdf).

Using the formulae presented in TIGR's SOP #M004, calculate the total pmol of synthesized cDNA, the total pmol of incorporated Cy dye for each labeling reaction, and the nucleotide/dye ratio for each reaction.

Optimal labeling reactions generate >2000 pmols of cDNA, >150 pmol of Cy dye, and a nucleotide/dye ratio that is <50.

For each Cy-labeled cDNA sample that will be used in a two-color (competitive) hybridization, calculate the volume of Cy5-labeled cDNA and Cy3-labeled cDNA that is equivalent to 50 pmol of incorporated Cy5 and 50 pmol of incorporated Cy3.

Combine the volumes calculated in step 3 in a single microfuge tube and dry the combined cDNA targets in a roto-evaporator (45oC).

Resuspend the combined and dried cDNA targets in 70ul of Corning Universal Hybridization Solution.

Incubate re-suspended cDNA targets at 95oC for 5min

Centrifuge at max speed for 1 minute (room temperature).

Clean "blocked" slides by blowing compressed air passed through a 0.2 □m filter disk and then place array in a Genomic Solutions (or similar, e.g. Corning) Hybridization Chamber; fill humidity wells as per the instructions that come with the hybridization chambers (I use 20 ul) .

Clean LifterSlipsTM by blowing compressed air passed through a 0.2 □m filter disk

Carefully cover the array with a clean dry LifterSlipTM.

Carefully pipette 65ul of re-suspended cDNA targets to the edge of the LifterSlipTM, allowing the solution to cover the array by capillary action.

Seal the array chamber (without moving the LifterSlipTM) as per the instructions that come with the hybridization chambers.

Incubate the sealed chamber containing array at 43oC for 12Â–16 hours. Conduct the hybridization in the dark.

Washing Arrays after Hybridization

Fill two foil-covered Coplin jars with pre-heated Wash Solution #1.

Remove the LifterSlipTM from the array surface by dipping arrays in the first Coplin jar containing Wash Solution #1; the LifterSlipTM should slide off the array easily.

Place the array in the second Coplin jar containing pre-heated Wash Solution #1.

Incubate arrays in Wash Solution #1 for 10 minutes at 43oC.

Transfer arrays to a new foil-covered Coplin jar containing Wash Solution #2.

Incubate arrays in Wash Solution #2 for 10 minutes at room temperature Agitate arrays gently during wash step.

Transfer arrays to a new foil-covered Coplin jar containing Wash Solution #3.

Incubate arrays in Wash Solution #3 for 10 minutes at room temperature Agitate arrays gently during wash step.

Immediately dry the washed arrays via gentle centrifugation, as described above. Do not let the arrays dry out prior to the centrifugation step.

1 minute at 1000-1500 × rpm

Place arrays in foil-covered slide tray until scanning.

Scanning Arrays We scan our arrays immediately after they are washed/dried using a two-channel confocal microarray scanner (ScanArray5000; GSI Lumonics, MA) and the associated ScanArray software (v3.1, Packard BioChip Technologies, MA). After laser focusing and balancing of the two channels, scans are conducted at a resolution of 10 um with the laser power typically set between 90-100% of maximum and the photomultiplier tube typically set at 65-75% of maximum. Excitation/emission settings are 543 um/570 um and 633 um/670 um for the Cy3 and Cy5 fluors, respectively. Raw fluorescence image data is saved as .tif files.

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BIOGRAPHICAL SKETCH

Gogce Ceren Kayihan was born in Ankara, Turkey in 1975. She studied in Amasya until the end of her high school education. In 1993, she entered Biological Sciences Department in Middle East Technical University. Upon her graduation in 1997 she started working on her master's thesis in the same department with her advisor, Dr. Zeki Kaya. Her love of plants and his research interest in conifers ended her up in a project where she studied genetic variation of Cedrus libani populations in Turkey. In 2001 she was accepted to the School of Forest Resources and Conservation at the University of Florida as a PhD student and started to work with Dr. Timothy White and Dr. John Davis.