Title: The role of tomato domestication in the quantitative genetic basis of Botrytis cinerea virulence

Abstract:

Most cloned naturally variable plant genes that provide pathogen resistance have large phenotypic effects that elicit qualitative resistance, dependent upon presence or absence of Avr or molecular pattern genes in the pathogen. Further, most of these resistance genes have been studied using epidemic pathogens. In contrast, most pathogens are endemic with a largely quantitative genetic architecture of host resistance which depends upon the genotypes of both host and pathogen. *Botrytis cinerea*, an endemic necrotroph, has the capacity to infect nearly every plant species and provides a unique opportunity to study how the virulence networks of a single pathogen can evolve to attack all plants.

We are beginning a GWA approach to understand how a single fungal species can have this broad host range. Full genomic sequencing of 96 *B. cinerea* genotypes showed that *B. cinerea* has the highest recorded level of standing genetic variation within plant pathogens. This broad host range is likely facilitated by recombination amongst highly polymorphic genes within a massive population. Variation in virulence interactions between *B. cinerea* and its plant hosts is entirely quantitative with no evidence of qualitative variation, in contrast to the majority of plant pathogens studied.

To identify which pathogen genes vary to target diverse plant hosts, we examined quantitative resistance of diverse tomato hosts in response to the 96 isolates of *B. cinerea*. We associate lesion variation on six domesticated lines and six close wild relatives with genetic variation in the 96 *B. cinerea* isolates. A first-pass GWA analysis in tomato (*S. lycopersicum* and *S. pimpinellifolium*) is complete, and we will present our preliminary observations on the quantitative interactions of the host and pathogen’s genomes. We are quantifying the genetic contribution to lesion size of variation in *B. cinerea*, tomato, and the interaction between these genomes. Using the collection of domesticated and wild tomatoes, we are also testing if there is a signature of domestication in how tomato responds to all or a subset of *B. cinerea* isolates.

Background / Introduction:

* For generalist necrotrophs, disease (virulence?) is a quantitative trait (ref 74 JAC 2016)
  + / quantitative resistance *studied from the pathogen side*
    - *QR in botrytis*
  + Most plant immunity studies on large-effect genes (qual-R)
* Applied goal to control common, economically costly pathogen
* Previous knowledge of quantitative genetics of virulence in Botrytis
  + Jason’s GWAS
    - Botrytis x At as model of quant R
  + Known genes for virulence
* Multigenic basis of plant resistance to Botrytis
  + Genetics in Arabidopsis – Jason’s GWAS
  + Mutant analysis in Arabidopsis
* Approach: detached-leaf GWAS using genetics of Botrytis
  + Can extend to additional host species
* Domestication in pathogen resistance
  + Theory: selection against defense alleles in domestication & cultivation
  + Assumes: low resistance in all domesticated varieties
* Questions
  + Does domestication give us a strong hypothesis about susceptibility?
    - Does this differ depending on: pathogen genotype, host genotype
  + Genetic basis of virulence in Botrytis
    - Do the same loci confer virulence across host genotypes?
* Summary of findings
  + Number of genes associated with phenotype
  + GO terms associated with phenotype
  + Conditionality by host species
  + Validation – AFTER all host spp studied

Most plant immunity studies have focused on large-effect genes controlling qualitative resistance.

The extent of infection is a cumulative phenotype, including both the virulence of the pathogen, and the susceptibility of its host. Both of these traits are highly quantitative, due to environmental and developmental factors, as well as complex genetics.

Gene-gene resistance is common in the plant immune response to biotrophic pathogens, but these same pathways provide no resistance advantage in response to necrotrophs; as such, plant resistance to necrotrophs is largely quantitative (ref 74 JAC 2016), controlled by many small-effect loci.

Further, if the pathogen population is diverse, the pathogen can rapidly evolve to respond to large-effect loci in its plant hosts. This makes quantitative resistance the only likely option for plant defenses against generalist pathogens.

We are interested in the genetic basis of plant susceptibility and pathogen virulence. Botrytis cinerea infects *over 200 plant species including all eudicots, and members of Pinus and Physcomitrella.* We are studying how Botrytis cinerea genetic variation controls both quantitative resistance and quantitative virulence.

* Applied goal to control common, economically costly pathogen
* Previous knowledge of quantitative genetics of virulence in Botrytis
  + Jason’s GWAS
    - Botrytis x At as model of quant R
  + Known genes for virulence
* Multigenic basis of plant resistance to Botrytis
  + Genetics in Arabidopsis – Jason’s GWAS
  + Mutant analysis in Arabidopsis
* Approach: detached-leaf GWAS using genetics of Botrytis
  + Can extend to additional host species
* Domestication in pathogen resistance
  + Theory: selection against defense alleles in domestication & cultivation
  + Assumes: low resistance in all domesticated varieties
* Questions
  + Does domestication give us a strong hypothesis about susceptibility?
    - Does this differ depending on: pathogen genotype, host genotype
  + Genetic basis of virulence in Botrytis
    - Do the same loci confer virulence across host genotypes?
* Summary of findings
  + Number of genes associated with phenotype
  + GO terms associated with phenotype
  + Conditionality by host species
  + Validation – AFTER all host spp studied

“Previous study of GWA of Botrytis lesion size to the genome of Arabidopsis found several cellular functions overrepresented. These included R-genes (RLKs and NLRs), genes previously identified in controlling At resistance phenotypes to Bc (RLM3, RST1,NPR1, VSP2, ATG2, PAD4, LYK3, COI1, LYK4, Erecta (ER), AXR1, and BOI) (JAC 2016 - ref 55, 56), defense response, oxidation-reduction processes,

and nucleic acid mismatch repair mechanisms, callose deposition, methylation-dependent chromatin silencing, transmembrane transport, mismatch repair mechanisms and categories related to modification of the cell wall (JAC 2016 – S10 fig, S6 Table)”

Materials & Methods

* Plant growth & choice of accessions
* Pathogen propagation & population of isolates
  + Number of genes in Bc genome
  + Number of SNPs at MAF > 0.20 (plus MAF > 0.10 / > 0.05?)
    - Number of genes with at least 1 SNP
    - Number of genes with >= 2 SNPs
  + Check for population structure
* Detached leaf assay
  + Whole-plant translatable
  + Lesion size as approximation of virulence
* Linear models
* Genome
* bigRR
  + JAC GWAS
* gene identification

Methods

Tomato genetic resources

We obtained seeds for 12 selected tomato genotypes in consultation with Dr. Roger Chetelat at the UC Davis TGRC. These include a diverse sample of 6 genotypes of domesticated tomato’s closest wild relative (S. pimpinellifolium) from throughout its native range (Peru, Ecuador) as well as 6 heritage and modern varieties of S. lycopersicum. We bulked all genotypes in long-day (16h photoperiod) greenhouse conditions at UC Davis in fall 2014. Plants were grown under metal-halide lamps using day/night temperatures at 25°C/18°C in 4” pots filled with standard potting soil (Sunshine mix #1, Sun Gro Horticulture). Plants were watered once daily. Plants were pruned and staked upright, and fruits were collected as they matured.

Fruits were stored at 4°C in dry paper bags until seed cleaning. Seeds and locule contents were incubated at 24°C in 1% protease solution (Rapidase C80 Max) for 2h, then rinsed in dI H2O and air-dried. Seeds were then stored in a cool, dry, dark location until further plantings.

We bleach-sterilized all seeds prior to germinating on germination paper in growth chambers. At 7 days we transferred seedlings to soil (SunGro) and grew all plants in growth chambers in 20°C, short-day (10h photoperiod) conditions with 180-190 uM light intensity and 60% RH. The flat was covered with a humidity dome during germination. We bottom-watered with dI H2O every two days for two weeks, and at week 3 watered every two days with added nutrient solution (0.5% N-P-K fertilizer in a 2-1- 2 ratio; Grow More 4-18-38). Plants were used for detached leaf assays 6 weeks after seedlings were transferred to soil.

Botrytis genetic resources

[Selection of genotypes / population collection]

Botrytis growth

Botrytis isolates were maintained as conidial suspensions in 30% glycerol for long term storage at -80°C. For regrowth, spore solutions were diluted to 10% in 50% filter-sterilized grape juice, then inoculated onto 39g/L potato dextrose agar (PDA) media. Isolates were grown at 25°C in 12h light, and propagated every 2 weeks.

Detached leaf assay

To study the effect of genetic variation in host and pathogen on lesion formation, we infected detached leaves of 12 diverse tomato varieties with the above 96 Botrytis isolates. We used a randomized complete block design for a total of 6 replicates across 2 experiments. Leaflets were placed on 1% phytoagar in seed flats, with humidity domes on top.For each plant genotype, leaflets from each of 10 plants were placed onto agar in blocks. Leaves were selected by a random sample of 5 leaves per plant, and 2 leaflet pairs per leaf.

Spores were collected from mature (1-2 week old) Botrytis cultures, and diluted to 10 spores/ uL in 50% filter-sterilized grape juice. 4ul droplets of spore suspensions were inoculated onto detached leaves at room temperature with 24h light. Control leaves were mock-inoculated with 4uL of grape juice without spores.

We took digital photos of all leaflets at 24, 48, and 72 hours post inoculation for downstream image analysis.

Automated Image Analysis

We measured lesion areas using the EBImage and CRImage packages (Pau et al., 2010; Failmezger et al., 2010) in the R statistical environment (R Development Core Team and Team, 2009). Leaflets were identified as objects with green hue, and lesions were identified as low-saturation objects within leaves. Images masks were generated for both the leaf and lesion, then manually refined by a technician to ensure accurate object calling. The area of these leaves and lesions were then automatically measured as pixels per lesion and converted to area using a 1 cm reference within each image.

Data analysis

We analyzed by F-test the generalized linear model for the full experiment, including the fixed effects of isolate, plant domestication, plant genotype (nested within domestication), the random effects of experiment, and the interaction effects of experiment with isolate and experiment with plant.

We calculated the least-squared means of lesion size within each tomato genotype. We included the fixed effect of isolate, and the random effects of experiment, the isolate by experiment interaction, and leaflet pair (nested within leaf, nested within individual plant). We then used these means as the phenotype input to our custom bigRR script for GWA. We used SNPs from (*get SNP details from Suzi*). Because bigRR provides an estimated effect size, but not a p-value, we perform permutation analyses to determine effect significance. We permute the phenotypes 1000x and re-run bigRR, to establish 95%, 99%, and 99.9% thresholds for significance.

Results

* variation in lesion size
  + domesticated lines more susceptible on average BUT overlap with wild
  + analysis of GLM
  + spearman’s rank correlation among phenotypes
    - is there a higher correlation in lesion size across isolates for domesticated or wild varieties?
  + Proportion of variance for isolate genotype *across plant genos* – barplot
    - Variance determined using a standard guassian linear model where Phenotype = Experiment + Experiment(Plate) + Isolate for each plant geno.
* genetic control of lesion size
  + due to plant, pathogen, and INTERACTION
    - variation between Bc genotypes affects virulence on tomato
    - how best to draw interaction plots?
    - Are high-virulence isolates on tomato also virulent across hosts?
      * Where does B05.10 (most common for study) fall?
* genes for lesion size
  + number of significant loci > threshold
    - phenotype-conditional loci (single host genotype)
    - total loci (sum across phenotypes)
    - domestication-conditional loci?
    - quantitative resistance to botrytis depends on both plant and pathogen genetics
  + quantitative – justifies study beyond single-pathogen level.
    - What is the highest MAF for our significant SNPs? 🡪 approximate minimum population to detect the low-hanging fruit
  + SNPs for previously-IDed pathogenesis genes not found in our GWAS list?
  + SNPs unique to each plant host? // shared?

Results

Interaction of genetic diversity in B. cinerea and tomato

\*\* violin plot domesticated vs. wild

Overall, domesticated varieties show higher susceptibility to diverse B. cinerea isolates (Table R1) (Figure R0), and the three highest-susceptibility varieties are within S. lycopersicum. However, one domesticated variety, LA4345 (Heinz 1706) is relatively resistant to B. cinerea isolates even compared to wild varieties.

Figure R2. Lesion area for each isolate by tomato plant interaction. Tomato accession names are listed, and each isolate is traced by a single color. Virulence of isolates changes across tomato genotypes within both the domesticated and wild groups.

Susceptibility is higher overall in tomato hosts compared to *A. thaliana* (Figure R3). virulence of several isolates changes across host species (Figure R4).

Factors in lesion size phenotype

Table R1. Mixed model analysis of lesion area. Interaction ( : ) and nesting ( / ) of terms are included. Lesion size is significantly affected by pathogen and host genotypes, and domestication status. However, genotype effects differ across the two replicate detached leaf experiments.

F-test from a GLM of lesion size revealed a significant effect of B. cinerea genotype, tomato genotype, and tomato domestication on lesion size. The interactions between B. cinerea and tomato genotypes were non-signficant under our model (\*\* interpretation). The F-test also revealed significant effects of experimental replicates and flat blocking, which we were able to account for in the model. The effect of individual plant was non-significant for lesion size (\*\* interpretation).

There was a statistically significant interaction between XX and YY for lesion size (interpretation).

Given the effect of host genotype on lesion size, we conducted GWA analysis for lesion size individually within each host.

Interspecific comparisons

Consistent genetic basis of lesion size between tomato and Arabidopsis?

Any shared SNPs/ gene models?

Shared networks? … network based on SNP co-occurrence between plant/ isolate genotypes

How many isolates remained in same “rank category” across species?

How many isolates were saprophytic on Arabidopsis but virulent on tomato?

Genetics of isolates with “strong” rank-order shift?

Genetics

For top 50 SNPs/ geno:

* Table of effect-size estimates per association
* Table of MAF per association

Table R1. Mixed model analysis of lesion area. Interaction ( : ) and nesting ( / ) of terms are included. Lesion size is significantly affected by pathogen and host genotypes, and domestication status. However, genotype effects differ across the two replicate detached leaf experiments.

Figure R0. Violin plot of lesion size on domesticated vs. wild tomato hosts.

Figure R1. Average lesion area on each tomato plant genotype, across all *B. cinerea* isolates. Tomato accession names are listed.

Figure R2. Lesion area for each isolate by tomato plant interaction. Tomato accession names are listed, and each isolate is traced by a single color. Virulence of isolates changes across tomato genotypes within both the domesticated and wild groups.

Figure R3. Variation in lesion area for each *B. cinerea* isolate, averaged across plant host genotypes. Lesion size data from *S. lycopersicum* and *S. pimpinellifolium* is compared to the same set of isolates on *A. thaliana*.

Figure R4. Variation in lesion area for each *B. cinerea* isolate, averaged across plant host genotypes. Lesion size data from *S. lycopersicum* and *S. pimpinellifolium* is compared to the same set of isolates on *A. thaliana*. Each isolate is traced by a single line color. Relative virulence of several isolates changes across host species.

Discussion:

* Next steps:
  + condense loci into co-expression networks
    - In unpublished data, we infected 96 diverse B. cinerea isolates onto wildtype Col-0 A. thaliana and examined lesion phenotypes in planta, as well as the transcriptome of infected leaves. At 24 hours post-infection (hpi), PCA revealed that the predominant vector of transcripts could predict lesion size variation at 72 hpi with >70% accuracy for all pathogen genotypes and across multiple plant genotypes. This vector was not controlled by SA or JA signaling, suggesting novel pathway(s) in the model of B. cinerea and host signaling.
  + Additional host species
    - Genetics of virulence conserved/ variable?
    - patterns of domestication

Acknowledgements/ Authorship\*:

Detached leaf assay: Baohua Li, Aysha Shafi, Dick Leon, Dihan Gao, Ayla Nelson, Hart Caligagan, Gongjun Shi, Raoni Gwinner

Image Analysis: Dick Leon, Dihan Gao

Statistical Analysis: Rachel Fordyce, Raoni Gwinner, Suzi Atwell, Jason Corwin

Writing: