**Natural variation for Botrytis cinerea virulence and susceptibility of domesticated and wild tomato**

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**Introduction**

* For generalist necrotrophs, virulence & susceptibility are quantitative traits
  + Most plant immunity studies on large-effect genes (qual-R)
* genetics of Botrytis virulence
  + virulence loci on tomato
  + Quantitative genetics of resistance in Arabidopsis – Jason’s GWAS
* Domestication in pathogen resistance
  + Theory: loss of resistance alleles in domestication & cultivation
    - Email JRI for assumptions in maize genomics
  + Assumptions
    - domestication model holds for disease resistance
    - Resistance is low across all domesticated varieties
    - applies to plants domesticated for fruit
* Approach: detached-leaf GWAS using genetics of Botrytis
  + Work with variation in pathogen
  + Can expand to additional host species - generalist
* Questions
  + Does domestication give us a strong hypothesis about susceptibility?
    - Is this dependent on: pathogen genotype, host genotype
  + Genetic basis of virulence in Botrytis
    - Do the same loci confer virulence across host genotypes?
* Summary of major findings
  + Contributions of host, domestication, isolate and interactions
  + Highly quantitative: Number of SNPs/genes associated with phenotype
  + GO terms associated with multi-host candidate genes

**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**

**Experimental Design**

We wanted to directly measure the impact of tomato domestication and genetic variation on quantitative resistance. To measure quantitative resistance, we infected tomato leaflets with a collection of 91 diverse *B. cinerea* isolates. *B. cinerea* is an endemic necrotroph, and host resistance to this generalist pathogen is quantitative, with no evidence of qualitative defense loci (Rowe and Kliebenstein , Corwin, Copeland et al.). Previous studies have examined the contrast in *B. cinerea* resistance between wild and domesticated tomato using distantly related species such as *S. chilense* (Nicot, Moretti et al. 2002, Ten Have, van Berloo et al. 2007), *S. chmielewskii* (Nicot, Moretti et al.), *S. habrochaites* (Finkers, van Heusden et al. 2007, Ten Have, van Berloo et al. 2007), *S. hirsutum* (Egashira, Kuwashima et al. 2000, Nicot, Moretti et al.), *S. lycopersicoides* (Guimaraes, Chetelat et al. 2004), *S. neorickii* (Ten Have, van Berloo et al. 2007, Finkers, Bai et al. 2008), *S. peruvianum* (Egashira, Kuwashima et al. 2000, Nicot, Moretti et al.), *S. pennellii* (Nicot, Moretti et al.) and *S. pimpinellifolium* (Egashira, Kuwashima et al. 2000, Nicot, Moretti et al. 2002). These single-isolate studies found a wide range of pathogen susceptibility levels both within and between tomato species, though none of the studies directly compared wild versus domesticated genotypes. We selected *S. pimpinellifolium*, the closest wild relative of *S. lycopersicum*, to directly study the selection associated with the impact of domestication. We selected tomato genotypes including 6 domesticated *Solanum lycopersicum* cultivars and 6 wild *S. pimpinellifolium* genotypes. We infected all 91 *B. cinerea* isolates onto each plant genotype in 3-fold replication across 2 independent experiments in a randomized complete block design, giving 6 measurements per plant-pathogen combination, for a total of 3,276 lesions. We digitally measured the area of the developing lesion at 72 hours post infection (HPI) (Figure R1). At 72 hours, significant lesion growth was visible, but no lesions had grown to completely consume infected leaflets. Lesion area is a composite phenotype from the interaction of host and pathogen genetics that has been utilized in a number of studies on the molecular and quantitative genetic basis of plant-*Botrytis* interactions (Rowe and Kliebenstein).

We performed statistical analysis of lesion size with a generalized linear model (GLM). Within the model, we tested the fixed effects of isolate genotype, plant species (domesticated or wild), plant genotype (which is nested within species), and position of sampled leaflet (apical or basal). We also considered the random effects of experiment, block (nested within experiment), individual plant, and individual leaf (nested within sample plant). The terms for individual plant, leaf, and leaflet position did not significantly improve the model, so we omitted them from further analysis. Our final model also included the interaction terms of isolate by plant species, and isolate by plant genotype (nested within species). The final model shows that genetic variation within both the host plant and the pathogen affect lesion growth (Table R1). Domestication also impacted lesion formation, as shown by the significant effects of tomato genetic variation between domesticated and wild species. We did not find evidence for significant interaction effects between isolate and plant genotypes. This may have been due to the vast number of degrees of freedom, as the isolate-plant interactions contribute a large proportion of the variance in lesion size (Table R1).

**Domestication and lesion area**

Existing literature, largely studying qualitative resistance to biotrophic pathogens, has proposed that domestication increases susceptibility to pathogens and decreases plant genetic variation for disease resistance due to selection bottlenecks during domestication.

In agreement with domestication theory, lesion size is slightly greater on average (18% increase) on domesticated tomato compared to wild tomato (p = 0.058, Table R1) (Figure R2).

In contrast to theory, the domesticated tomato genotypes had a wider range of average lesion formation than wild genotypes (5% to 95% interval: 2.03 cm2 range on domesticated, 1.76 cm2 range on wild). A domestication bottleneck would lead to reduced variation for lesion size across domesticated tomato genotypes; instead we observe an increased range of lesion sizes in domesticated compared to wild tomato. Additionally, the ordering of isolates by coefficient of variation (CV) of lesion size does not statistically differ between domesticated and wild hosts (Wilcoxon signed-rank test, V=2275, p=0.7163), indicating a lack of evidence for a domestication effect on lesion size variance (Figure R3). Overall, we see evidence for a slight domestication impact on *Botrytis cinerea* defense, but this depends on host genotype.

**Host variation and lesion area**

Domestication does impact lesion size, but most variance is due to genetic variation in the isolate and in host plant genetics (Table R1).

**Pathogen**

Our 91 B. cinerea genotypes were isolated from various eudicot plant hosts, including tomato stem tissue (2 isolates; T3, KT) and tomato fruit (3 isolates; KGB1, KGB2, Supersteak)

Isolates collected from tomato stem or fruit tissue are not among the most virulent group (Figure R4F). For *B. cinerea* genotypes isolated from tomato tissue vs. other hosts, there is no significant difference in lesion size across all hosts on either domesticated (t-test; t=-1.10, 4.3 df, p=0.330) or wild (t-test; t=-1.09, 4.2 df, p=0.332) tomato. In fact, one isolate collected from tomato tissue (KGB1) is within the 10 least-virulent isolates (Figure R4F). This may suggest a generalist strategy for individual isolates, due to this apparent lack of host-specificity.

**Host-pathogen interactions**

**Domestication**

We also tested whether genetic differences between wild and domesticated hosts interact with pathogen genetics to determine lesion size. Lesion size varies across host for many of the isolates, suggesting an interaction between the genomes of *B. cinerea* and tomato (Figure R4). However, domestication did not have a significant interaction effect with isolate genotype (Table R1). This is likely due to the many degrees of freedom in calculating this interaction effect. As such, we took alternate approaches to examine the interaction between domestication and isolate in determining lesion size. We performed ANOVA for each isolate examining the fixed effects of plant and domestication, and the random effect of experiment. Following FDR correction for multiple testing, three isolates showed a significant effect of domestication on lesion size (Figure R4F). These included one of the highly virulent isolates, an intermediate isolate, and one of the saprophytic isolates, suggesting that the domestication effect is not dependent on isolate virulence.

Isolate ranking by mean lesion size differs between domesticated and wild hosts (Wilcoxon signed-rank test, V=4322, p=2.586e-12) (Figure R3).

**Plant genotype**

Tomato genotype within each species did not have a significant interaction effect with isolate genotype (Table R1). The F-test could identify significant effects of X,Y,Z but not the interaction between them, because F-tests with high numbers of degrees of freedom can be significantly underpowered, like in the case of the isolate x plant genotype interaction term (df: 940). We took an additional approach to statistically test for an interaction between *B. cinerea* and host genotype. We split the dataset by isolate, and within each new dataset performed GLM ANOVA with the fixed effects of domestication and plant genotype nested within domestication, and the random effect of experiment. Through this single-isolate GLM analysis, a subset of isolates show a significant (p < 0.05) interaction with host genotype (Figure R4E).

**BigRR/GWAS**

* brief description of methods
* Number of SNPs > thresholds (varies by tomato genotype)
* ~meta-analysis: SNPs that exceed 99.9% threshold across MULTIPLE host genotypes
* Genes <= 2kb from SNPS
  + GO terms
  + Clustering?
  + RNAseq on Arabidopsis

Questions:

* Add more connecting to Arabidopsis?
* Where to add heatmap (96 isolates x 12 tomatoes with Rows & Cols clustering

DISCUSSION

Cohesive argument = contrast generalist vs. specialists

* Domestication effects
  + Small effects
  + Domestication affects only a subset of 3 genotypes—justify working on full bc pop
  + Implication for breeding resistance: work w full pop
* No MAMPs/PAMPs identified
  + No chitin, glycans, glycolipids…
  + No mannans (JAC + Klieb)
* Not caused by a few genes of large effect
  + Effect size of SNPs small (~1 mm?)
  + MANY SNPs
  + Sum up SNP fx of top hits?
  + Test model using ~10 big SNPs for fx size on trait?

**FIGURES**

Figure R1. Will be an image of the detached leaf assay and leaf/ lesion calls.

Figure R2. Violin plots of lesion size due to Botrytis cinerea growth on tomato host genotypes. Plots include all replicates of lesion size measurements across isolates.

Figure R3. Interaction plot for domestication. Each line traces the average lesion size for a single Botrytis isolate.

Figure R4. Interaction plot of lesion size due to individual Botrytis cinerea isolates on tomato host genotypes. Each line traces the average lesion size across plant genotypes for a single Botrytis isolate.

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