CANDIDATE GENES FROM GWAS AND RNASEQ FOR BEECH BARK DISEASE RESISTANCE IN AMERICAN BEECH

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SUMMARY

American beech (*Fagus grandifolia* Ehrh.) is an aesthetically, ecologically, and economically important native component of the North American eastern hardwood forest. American beech is susceptible to beech bark disease (BBD), however, and has suffered high rates of mortality as the disease complex spreads. The invasive sap-feeding woolly beech scale insect (*Cryptococcus fagisuga*) is the pre-disposing factor for infection by introducing either *Neonectria faginata* or *N. ditissima* fungus species that result in extensive cankering of American Beech trees. A small percentage of trees survive BBD attack, and many of these show signs of natural resistance to the insect vector in the egg-inoculation tests used to assay for resistance. We have developed and applied genomics resources to learn more about the molecular genetic basis of gene expression and gene sequence variation associated with cases of natural resistance to the insect. Initially, transcriptome resources were developed, and differential gene expression analyses conducted from which candidate genes were selected. From the transcriptome, an SNP chip assay was developed to genotype an association population of 506 individuals from across the American beech range, 249 of which were resistant and 257 susceptible to BBD. We also constructed a genetic linkage map based on SNPs with a full-sib family of 115 individuals to locate BBD-resistance QTL. The GWAS project revealed four highly significant SNPs on Linkage Group 5 for a single gene encoding a metallothionein-like protein. Metallothioneins are cysteine-rich metal chelator proteins that can moderate oxidative stress by coordinating metal atoms, which may provide a resistance mechanism against the woolly beech scale insect.

OBJECTIVES

The primary goal of this research was to gain a better understanding of the molecular mechanisms underlying resistance and susceptibility to beech bark disease (BBD) in American beech. Our objectives towards this goal were to:

1. Develop genomics tools for molecular genetic studies in American beech.
2. Conduct differential gene expression analyses to identify candidate genes for future research on BBD resistance in beech.
3. Conduct a genome-wide association study (GWAS) to identify which genes or alleles have the greatest contribution to BBD resistance in natural stands of American beech.

METHODS AND RESULTS

Development of Genomics Tools for American Beech

Our development of genomic resources for American beech began with support from the National Science Foundation's Plant Genome Resources Program for the "Fagaceae Genomic Tool Development Project", which ran from 2006 to 2009. This project was led by Ronald Sederoff (North Carolina State University), with participants from Pennsylvania State University (John Carlson, Haiying Liang, Abdelali Barakat, Stephan Schuster), SUNY ESF (William Powell, Kathleen Baier, Charles Maynard), Clemson University (Albert Abbott, Margaret Staton, Jeff Tomkins,

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Steven Ficklin, Barbara Blackman, Eric Fang), North Carolina State University (Nick Wheeler, Chris Smith, Dahlia Nielsen, Ron Sederoff), The Connecticut Agricultural Experiment Station (Sandra Anagnostakis, Lila Pinchot), USDA Forest Service (Tom Kubisiak, Dana Nelson), The American Chestnut Foundation (Fred Hebard, Paul Sisco), and Science Advisory Board members (Doug Cook of UC-Davis, Jennifer Koch of USDA Forest Service, and Jeanne Romero-Severson of Notre Dame University).

The "Fagaceae Project" focused on Chinese chestnut (*Castanea mollissima*), American chestnut (*C. dentata*), Northern red oak (*Quercus rubra*), White oak (*Q. alba*), and American beech. For these five species we developed expressed sequence tag (EST) databases by next generation sequencing (NGS) with 454 technology (Roche) for use in gene discovery, DNA markers for genetic mapping, and in some species BAC libraries for gene cloning and physical mapping. In addition, work on high density genetic mapping and physical mapping was initiated. In total, 16 cDNA libraries were constructed and sequenced, including two for American beech. The beech libraries were prepared from poly-A RNA isolations from tissue collections designed to represent gene expression plant-wide, including roots, bark, buds, twigs, leaves, petioles, and flowers. The tissues were collected from USDA Forest Service genotype 1504—a healthy American beech tree, and genotype 1506—a BBD-infected American beech tree. For American beech, a total of 14.6 million bases of RNA sequence data (ESTs) were generated and assembled into 8,319 transcripts. From the assembled transcripts, 2,383 proteins could be predicted based on protein databases (such as InterProScan) of which 2,231 were assigned putative annotations and functions based on similarities to known genes in the NCBI databases. The EST sequences for each of the American beech trees 1504 and 1506 were deposited at the NCBI Short Read Database under accession numbers SRX001797 and SRX001798, respectively. The American beech EST sequences, transcripts, annotations, and DNA markers from the Fagaceae Project are also available for download and query at the Hardwood Genomics Database entry for *Fagus grandifolia* (https://www.hardwoodgenomics.org/organism/Fagus/grandifolia) that also includes a Gene Ontology Browser of 629 biological, 242 cellular, and 830 molecular functions predicted from the transcript annotations, as well as a KEGG Brower with the placement of the beech transcripts into enzymatic pathways.

After completion of the Fagaceae Project, genomic resources targeted to BBD-resistance in American beech were greatly expanded through a grant to Jennifer Koch from the USDA Forest Service Forest Health Protection's Special Technology Development Program. The project "Development of DNA-based markers to identify beech bark disease (BBD) - resistant trees in natural stands" ran from 2009 to 2012, with Jennifer Koch as Principal Investigator and participants from Pennsylvania State University (John Carlson, Donghwan Shim, Charles Addo-Quaye, Tyler Wagner, Lynn Tomsho), UC-Davis (David Neale, Irina Ćalić, Randi Famula, Mirko Ledda, Christopher Campbell), and USDA Forest Service Northern Research Station lab in Delaware, OH (David Carey).

The "BBD-resistance project" focused on gaining a better understanding of the molecular mechanisms underlying BBD-resistance in American beech. We studied a set of BBD-resistant and BBD-susceptible trees identified in natural stands by Forest Service researchers, some of which were accessioned as part of the beech bark disease genetic resistance improvement program (Koch et al. 2010; Houston and Houston 2000). RNA sequence data was produced by 454 NGS technology from each of 10 cDNA libraries prepared from poly-A RNA isolated from bark tissues of 5 BBD-resistant trees (USDA Forest Service accessions 1228, 1208, 2692, 1504, 2776) and from 5 BBD-susceptible trees (accessions 726, 3128, 1973, 2143, "Holden") following treatment of clonal replicates with larvae of the woolly beech scale under greenhouse conditions. The relative resistance of nine of the 10 clonal accessions (genotypes) (fig. 1) was assayed by determining the number of adult scale insects produced from genotypes following egg inoculations (the Holden genotype did not survive the inoculation test).

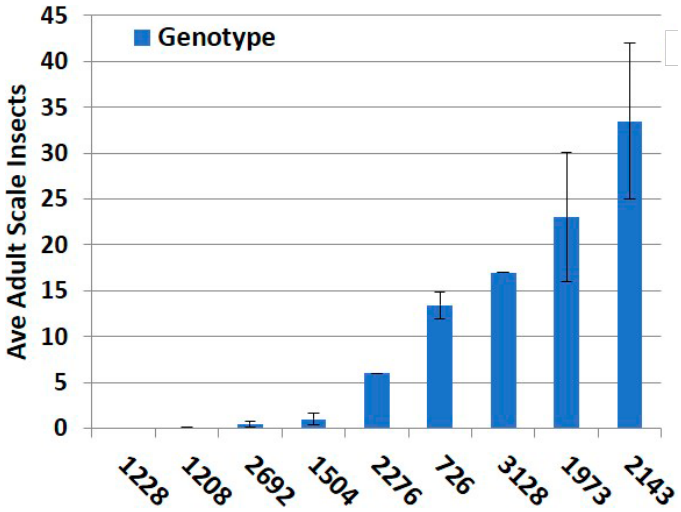

Figure 1—Average number of scale insects produced from clones of American beech genotypes following egg inoculation under greenhouse conditions. A range of resistance and susceptibility to BBD are demonstrated.

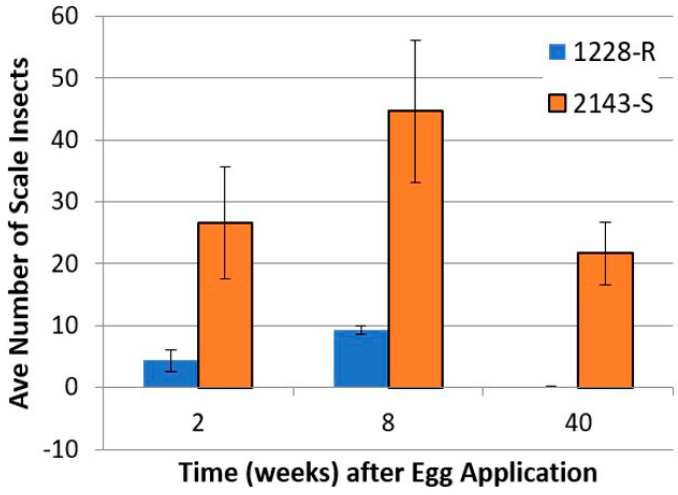

Figure 2—Comparison of scale insect development on BBD-resistant vs. BBD-susceptible genotypes 1228R and 2143S.

In total we obtained 257.5 Mb of RNA sequence data from the BBD-resistant trees and 508.7 Mb from the susceptible trees. The RNA sequences from the BBD-resistant trees were pooled as were the sequences from the BBD-susceptible trees, and then assembled into two sets of transcripts from which over 26,000 Single Nucleotide Polymorphism (SNP) DNA markers were identified for use in genetic linkage mapping and genome-wide association (GWAS) studies (Ćalić et al. 2017). The sequences from this study are available in the Short Read Archive at the National Center for Biotechnology Information, genotype numbers SRX1781388 to SRX1781397 (NCBI BioProject genotype PRJNA321730; http://www.ncbi.nlm.nih.gov).

From the 10 beech tree genotypes, we chose genotypes 1228 ('1228R') and 2143 ('2143S') to represent the putatively most BBD-resistant and BBD-susceptible (fig. 1) for an initial study of the response of gene expression in beech to attack by the scale insect. The results of a time course comparing insect viability at 2, 8, and 40 weeks, confirmed the pronounced differences in susceptibility between genotypes 1228R and 2143S (fig. 2).

The workflow devised for analysis and comparison of gene expression in genotypes 1228R and 2143S is presented in figure 3. Steps completed during this project are indicated by solid lines, while dotted lines suggest steps that should be taken in future projects, based on results gained in the BBD-resistance project.

The RNA sequence yields and statistics from the cDNA libraries from genotypes 1228R and 2143S are shown in table 1. A total of 1,021,530 sequence reads corresponding to 403,362,357 bases were obtained from the two outer bark tissue RNA samples. The RNA sequences from the two libraries were assembled separately and after pooling (using Trinity software). A total of 22,463 transcripts were assembled for genotype 1228R and 21,957 for genotype 2143S, and 31,525 from the pooled RNA sequence data.

Of the 31,525 transcripts in the reference set of transcripts from pooling the two sets of RNA sequences prior to assembly (table 1 "Both"), 26,784 transcripts were for unique genes (table 1 "Total trinity components"), while 4,741 were predicted to be isoforms from RNA-splicing. Of the 26,527 unique transcripts, 22,015 genes were expressed in both genotypes 1228R and 2143S, while 2,352 transcripts could only be detected in genotype 1228R and 2,160 transcripts were unique to genotype 2143S (fig. 4A). The combined assembly served as a reference against which the number of individual sequence reads for each transcript from each library was aligned

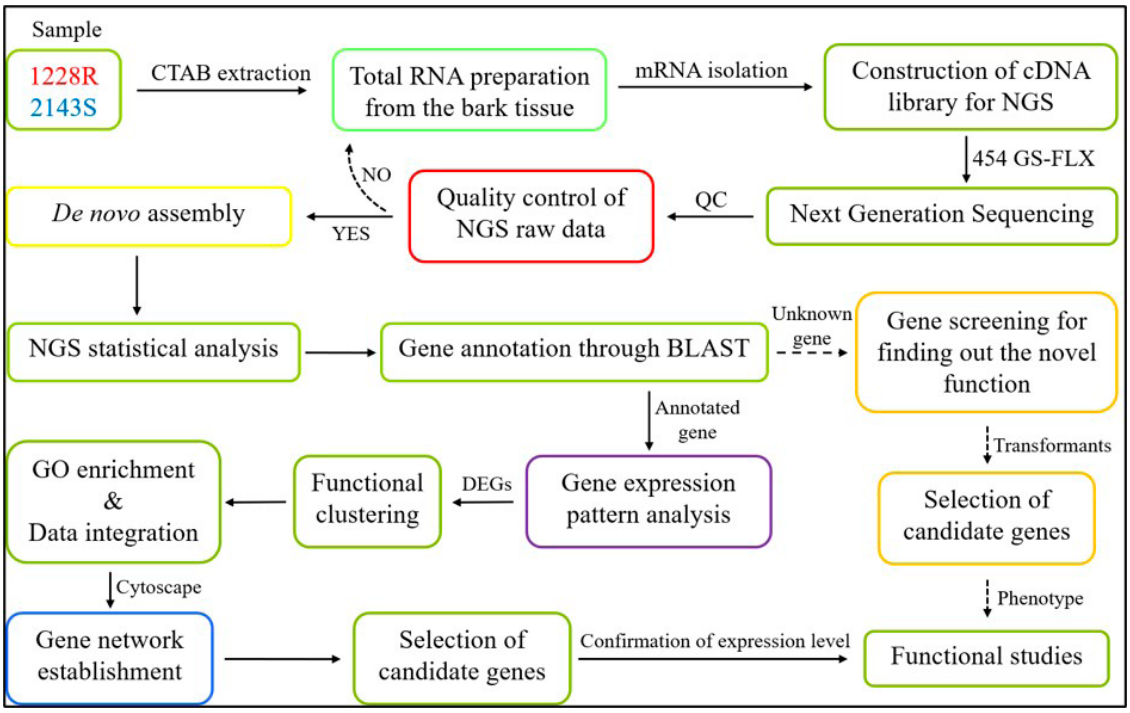

Figure 3—RNA sequence analysis workflow. Dashed arrows are steps which are recommended for future research projects.

Table 1—Statistics of 454 pyrosequencing reads and assembled contigs derived for BBD-resistant and BBD-susceptible *F. grandifolia* trees

		Resistant tree (1228R)	Susceptible tree (2143S)	
Raw reads				
	Num. sequences	530,622	493,807	
	Total nucleotides	211,316,501	191,666,186	
	Min. length	27	27	
	Max. length	1,568	1,558	
	Ave. read length	398.24	388.14	
	Q20	85.07	84.99	
	Q30	57.82	57.02	
	Ave. quality score	30.47	30.33	
Cleaned reads				
	Num. sequences	408,108	380,425	
	Total nucleotides	83,041,684	77,164,051	
	Min. length	50	50	
	Max. length	620	603	
	Ave. read length	203.48	202.84	
	Q20	98.1	98.15	
	Q30	87.62	87.11	
	Ave. quality score	36.29	36.14	
		Resistant tree (1228R)	**Susceptible tree (2143S)**	**Both**
Assembled contigs				
	Total trinity transcripts	22,463	21,957	31,525
	Total trinity components	20,574	20,238	26,784
	Percent GC	43.23	43.35	42.8
	Contig N50	754	735	921
	Average contig	606.89	599.17	688.78
	Total assembled bases	13,632,611	13,155,905	21,713,754

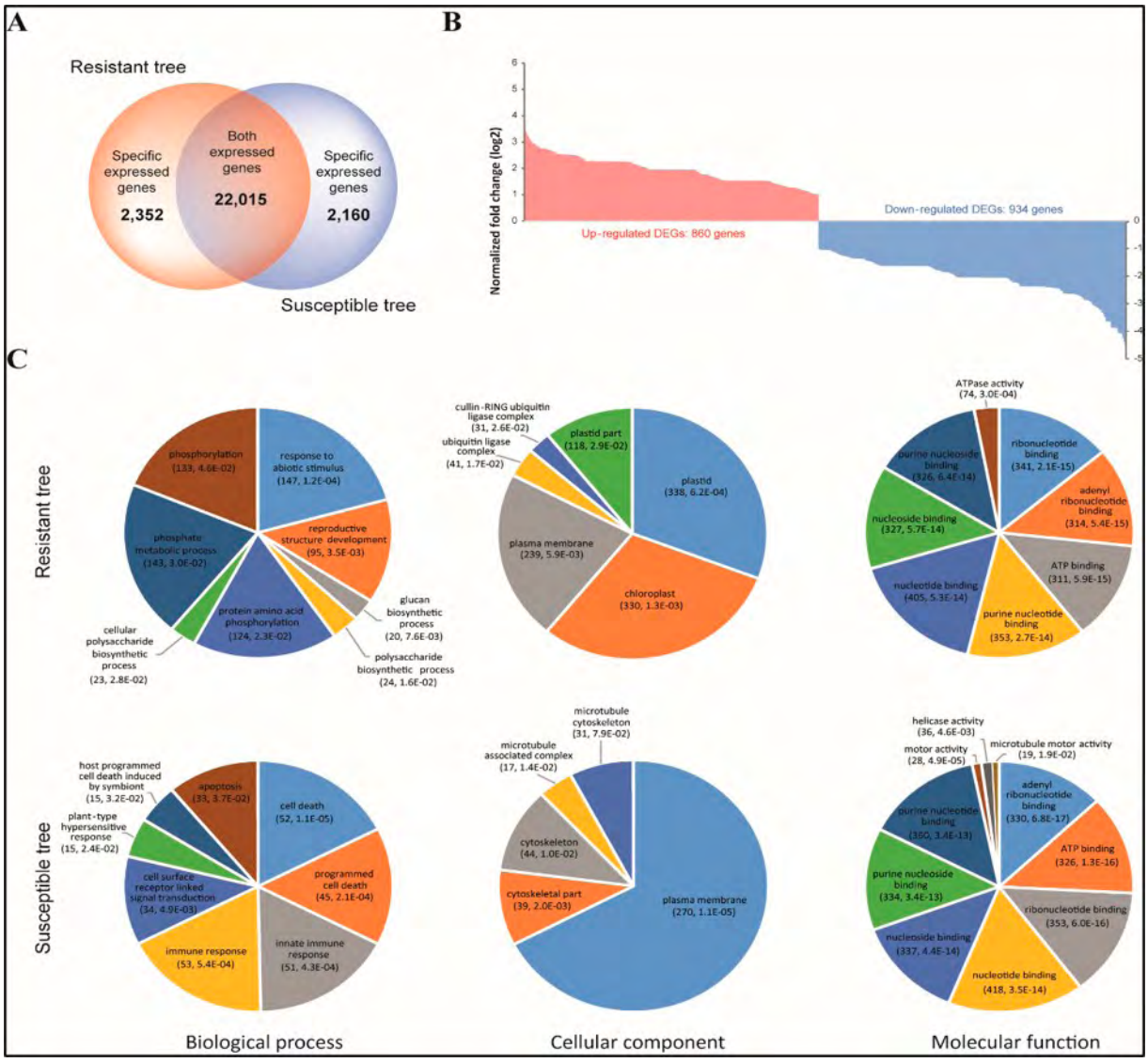

Figure 4—Distribution of differentially expressed genes (DEGs) in the BBD-resistant (1228R) and BBD-susceptible (2143S) American beech trees. (A) Venn diagram showing proportions of DEGs expressed in common and expressed uniquely in 1228R and 2143S trees; (B) Log-fold changes observed in up-regulated (induced) and down-regulated (suppressed) genes overall; (C) Venn diagrams showing numbers and proportions of Gene Ontology determined functional categories of DEGs in biological processes, cell components, and molecular functions.

and counted (using Qiagen/CLCBio Genomics Workbench software). Expression analysis of the RNA-Seq data revealed a total of 860 genes that were upregulated (induced) and 934 that were downregulated (suppressed) across the two genotypes, 1228R and 2143S (fig. 4B). Gene ontology (GO) analysis of putative functions showed a wide range of functional classifications represented in the transcriptomes of each genotype (fig. 4C). Twenty-two candidate genes, for research on BBD resistance in American beech, were selected based on the magnitudes of upregulation of expression in 1228R and alignment scores to known proteins (table 2).

To determine which of the 4,458 uniquely expressed transcripts were significantly different in expression between genotypes 1228R and 2143S, we conducted a differential gene expression analysis using the combined transcriptome referenced as above (using Qiagen/CLCBio Genomics Workbench software). A total of 608 genes were identified as significantly differentially expressed between genotypes 1228R and 2143S (at P-value < 0.01, unique reads > 5), of which 303 were up-regulated at least 2-fold and 305 were down-regulated by 2-fold or more. Among these differentially expressed genes (DEGs), 313 transcripts could be putatively identified by

Table 2—Candidate genes for BBD resistance in American beech based on relative strengths (fold change) of BBD-induced gene expression differences, rather than GO functional classifications. Only genes with the highest BLAST sequence alignment scores (E-values) to known genes in the model plants Arabidopsis and poplar in the NCBI Database are shown

Transcript ID	Lowest E-value	Accession (E-value)	AGI	NCBI annotation	Fold Change
AB_contig_394	8.732E-67	POPTR_0001s20780.1	AT2G04865.1	Aminotransferase-like, plant mobile domain family	8.165
AB_contig_1849	2.844E-130	POPTR_0016s14560.1	AT3G51550.1	Malectin/receptor-like protein kinase family protein	4.869
AB_contig_661	3.776E-156	POPTR_0010s18600.1	AT5G36890.1	Beta-glucosidase 42	4.217
AB_contig_2533	1.001E-124	POPTR_0002s03020.1	AT4G39090.1	Papain family cysteine protease	4.115
AB_contig_123	7.883E-27	POPTR_0001s03100.1	AT2G28910.1	CAX interacting protein 4	3.724
AB_contig_4199	1.516E-159	POPTR_0001s11360.1	AT2G46660.1	Cytochrome P450, family 78, subfamily A, polypeptide 6	3.5
AB_contig_5292	0	POPTR_0002s19480.1	AT3G62360.1	Carbohydrate-binding-like fold	3.335
AB_contig_4874	0	POPTR_0001s00330.1	AT1G63370.1	Flavin-binding monooxygenase family protein	3.126
AB_contig_25862	2.136E-22	POPTR_0001s20400.1	AT1G17720.2	Protein phosphatase 2A, regulatory subunit PR55	3.091
AB_contig_6118	4.385E-94	POPTR_0010s12440.1	AT1G23010.1	Cupredoxin superfamily protein	3.07
AB_contig_320	3.339E-109	POPTR_0001s17020.1	AT1G72650.2	TRF-like 6	2.923
AB_contig_2593	1.167E-106	POPTR_0002s07190.1	AT1G21360.1	Glycolipid transfer protein 2	2.885
AB_contig_5610	0	POPTR_0001s11860.2	AT5G49730.1	Ferric reduction oxidase 6	2.815
AB_contig_6174	1.285E-104	POPTR_0019s01650.1	AT5G17680.1	Disease resistance protein (TIR-NBS-LRR class), putative	2.759
AB_contig_8613	0	POPTR_0010s05170.1	AT1G60470.1	Galactinol synthase 4	2.38
AB_contig_2023	7.681E-67	POPTR_0011s12120.1	AT3G15360.1	Thioredoxin M-type 4	2.346
AB_contig_10163	7.492E-96	POPTR_0005s20870.1	AT1G17950.1	MYB domain protein 52	2.33
AB_contig_5378	9.648E-131	POPTR_0008s09140.1	AT1G13960.1	WRKY DNA-binding protein 4	2.154
AB_contig_9926	0	POPTR_0001s04500.1	AT2G42500.1	Protein phosphatase 2A-3	2.099
AB_contig_3528	2.117E-54	POPTR_0007s03840.1	AT5G09330.1	NAC domain containing protein 82	2.092
AB_contig_2070	1.343E-127	POPTR_0015s14600.1	AT4G22680.1	MYB domain protein 85	2.088
AB_contig_1218	0	POPTR_0003s21690.1	AT5G13000.1	Glucan synthase-like 12	2.003

homology to genes in the NCBI non-redundant protein database, while 295 transcripts were "unknown", i.e. with no sequence matches in the database.

The number of annotated (putative protein function) DEGs in the major GO functional classifications observed between the BBD-resistant vs. BBD-susceptible genotypes following greenhouse inoculations is shown by histogram comparisons in figure 5. In the BBD-resistant genotype 1228R, most DEGs were observed in functional categories related to stress-response - "response to abiotic stress" (Heidel-Fischer et al. 2018, Marwal et al. 2019, Tajima et al. 2020, Waterman et al. 2019), "polysaccharide and glucan related responses" (Piršelová and Matušíková 2013; Pogorelko et al 2013), "programmed cell death" (Locato and De Gara 2018), and "potassium ion transport" (Wang et al. 2013). In sharp contrast, the DEGs observed in the BBD-susceptible genotype 2143S were classified into GO functional categories that were in general more related to normal metabolic and developmental processes. These stark differences in gene expression in response to the scale insect indicate that genotype 1228R has the ability to recognize and mount a strong molecular defense against BBD, while genotype 2143S is susceptible due to lack of response at the gene expression level to BBD. We selected 17 candidate genes (table 3) for future studies, based on the four GO functional classifications and the putative gene assignments of DEGs observed in BBD-response by the resistant genotype.

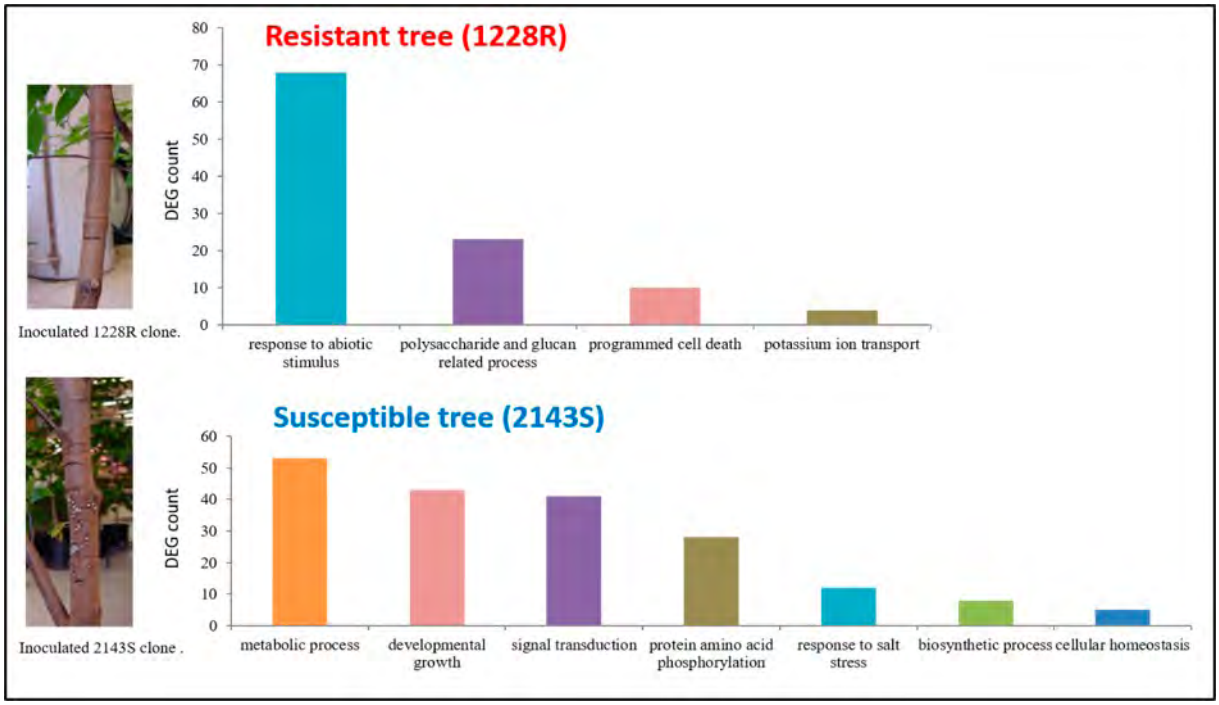

Figure 5—Gene Ontology functional categories of differentially expressed genes.

Table 3—Gene Ontology predicted descriptions for differentially expressed candidate genes in the stress response and apoptosis functional categories

Term	Description	Count	P-value
GO:0009628	response to abiotic stimulus	34	2.21E-04
GO:0048585	negative regulation of response to stimulus	6	2.79E-03
GO:0055114	oxidation reduction	29	6.53E-03
GO:0009266	response to temperature stimulus	11	3.29E-02
GO:0009416	response to light stimulus	13	3.80E-02
GO:0009414	response to water deprivation	7	4.29E-02
GO:0009314	response to radiation	13	4.70E-02
GO:0005976	polysaccharide metabolic process	10	4.55E-03
GO:0000272	polysaccharide catabolic process	5	2.21E-02
GO:0044042	glucan metabolic process	7	2.23E-02
GO:0009250	glucan biosynthetic process	5	2.31E-02
GO:0006073	cellular glucan metabolic process	6	3.38E-02
GO:0012501	programmed cell death	10	5.12E-03
GO:0016265	cell death	10	1.17E-02
GO:0008219	cell death	10	1.17E-02
GO:0006915	Apoptosis	8	1.26E-02
GO:0006813	potassium ion transport	4	4.29E-02

Genome-Wise Association Study

The GWAS revealed four highly significant SNPs on Linkage Group 5 for a single gene encoding a metallothionein-like protein (Ćalić et al. 2017). Metallothioneins are cysteine-rich metal-chelating proteins. Metallothioneins and metallothionein-like proteins respond to abiotic and biotic stresses in plants, including heavy metals, insect herbivory and fungal infections (reviewed by Leszczyszyn et al. 2013). A major role of metallothioneins and metallothionein-like proteins is their anti-oxidant effects that moderate damage from oxidative stress through scavenging of cofactors needed by reactive oxygen generating enzymes. One of the largest groups of pathogenesis-related proteins are also upregulated after heavy metal treatment, suggesting cross-talk between the heavy metal (abiotic) and biotic stress responses in plants (Joshi et al. 2019). Metallothionein-like class II proteins have previously been identified in *Fagus sylvatica* (NCBI accession number CAA10232) and *Picea abies* (Yakovlev et al. 2006). Type 4 metallothionein-like protein genes were reported to be expressed in inner bark tissues of *Cryptomeria japonica* (Leszczyszyn et al. 2013).

CONCLUSIONS, CAVEATS AND FUTURE RESEARCH

It is important to point out that certain caveats existed with the GWAS approach taken in this study. First, at the time the GWAS study (Ćalić et al. 2017) was undertaken, and still at the time of this presentation and manuscript, a reference genome for American beech was not available. Thus, a SNP chip was used as the GWAS reference, based on the combined bark transcriptomes from BBD-resistant and BBD-susceptible trees, to target genes in the tissue and biotic stress of interest. However, unlike a full genome sequence, transcriptomes never include all possible genes of interest nor all the genome-wide variation that exists for a trait, especially outside of coding regions. For example, transcriptomes are limited to the specific time points selected to sample after treatment, which for practical reasons are always limited and cannot encompass all the cascades of gene expression that are qualitatively and quantitatively important in accomplishing resistance. In addition, when dealing with a complex disease such as BBD that involves an insect vector and multiple fungal pathogens, it cannot necessarily be presupposed that using a transcriptome reference based on only the insect establishment, feeding, and reproduction will provide any markers for resistance mechanisms to the fungal infection process. Furthermore, other loci or alleles that fall below a selected significance threshold may also collectively (quantitatively) be important in BBD-resistance, or important in specific populations but not across the range of populations sampled.

Validations of our GWAS results still need to be completed. Ideally, validation populations should be collected from the same or similar populations so that both resistant and susceptible individuals are included, but without advance knowledge of resistance prior to conducting the GWAS and not including samples used in the original study. This is a difficult criterion to meet when dealing with natural populations that are already experiencing mortality from BBD, and/or that are under other biotic or abiotic stresses affecting population structure.

Another powerful approach to validating GWAS results is to test whether the loci with significantly associated alleles map in or near QTL of the trait on genetic linkage maps. The Forest Service's beech bark disease genetic resistance improvement program is evaluating 118 individuals from the 1505 x 1504 full-sib family used to construct the SNP-based genetic linkage map in the GWAS study (Ćalić et al. 2017). At the time of this presentation, only part (46 trees) of the mapping population had so far been evaluated, for which two QTL for resistance were found at regions other than the metallothionein locus detected by GWAS. This negative result, if it holds up in the whole mapping population, does not necessarily invalidate the GWAS result which derived from the strongest genetic signal that could be detected in 327 genetically distinct individuals sampled from several populations, rather than inheritance from only two genotypes (parents of the mapping family which could be fixed at the GWAS locus). It could however suggest that multiple mechanisms may result in BBD-resistance, for

example constitutive resistance traits versus induced-resistance traits, and differences in the natural histories of population exposures to past environmental stresses.

The metallothionein-like protein gene detected in the GWAS study was not one of the candidate genes selected from our transcriptome studies following BBD treatments. Changes in expression of the gene are not necessarily expected from variants in the coding sequence. However, the transcriptome overall did contain 12 transcript contigs which aligned well by BLAST with two metallothionein protein genes in the plant model *Arabidopsis thaliana*—a metallothionein 2A gene (NCBI accession AT3G09390.1) and metallothionein 3 (NCBI accession AT3G15353.1). It would be interesting to conduct a qRT-PCR study of expression of metallothionein or metallothionein-like genes, and other candidate genes, during a detailed time course of response to inoculations of well-established resistant and susceptible genotypes. In the study on differential gene expression reported here, we directly compared only the two extreme genotypes. A detailed gene network analysis study including the 8 genotypes with intermediate resistance/susceptibility phenotypes might reveal additional candidate genes, perhaps of smaller individual effects, which might collectively still be quantitatively important. However, the ultimate validation of the metallothionein-like protein detected by GWAS will require functional genomics studies to confirm its role in BBD-resistance. That awaits development of a gene transfer system for American beech with which knock-out, suppression, or gene editing studies can be conducted.

Beyond basic knowledge of how beech trees respond to BBD, such transcriptome and GWAS studies aim to contribute to the restoration of the species by providing molecular tools for selection and breeding. Markers that exhibit a significant association with the resistance phenotype can provide the basis of efficient indirect selection techniques such as marker assisted selection (MAS) and genomic selection (GS). For simply inherited, single-gene resistance traits, having several DNA markers within the gene increases the power of MAS, as individuals within a population may vary as to how many or which of the markers they carry for the gene. The presence of several markers and thus possibly several alleles for resistance may also slow the breakdown of resistance that can be a concern with single-gene traits in long-lived organisms like American beech. It should be acknowledged, however, that individual markers or alleles for a trait obtained from QTL mapping or transcriptome studies with one family or a small number of genotypes may contribute to only a small amount of phenotypic variation within and between large natural populations (Korte and Fallow 2013). Thus, MAS conducted with a few markers may not accomplish substantial advancements of selection and breeding goals, if not incorporated into genome-wide approaches. Genome-wide selection models that aggregate the small effects of many markers or alleles for a wider range of traits have proven to be powerful in representing a substantial fraction of genetic and phenotypic variation in tree breeding programs (Resende et al. 2017a, 2017b; Müller et al. 2017; Tan et al. 2017). The costs and benefits should be carefully considered prior to undertaking such approaches, as costs (real and opportunity) may be prohibitive, particularly in trees of ecological value that lack commercial value.

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