Genetic diversity and qoi fungicide resistance

Study of genetic diversity and QoI fungicide resistance in frogeye leaf spot (*Cercospora sojina*) from Tennessee

Introduction

Frogeye leaf spot (FLS) of soybean (*Glycine max* Merr.), caused by the fungal pathogen *C. sojina* Hara, was first identified in Japan in 1915 and South Carolina, the United States in 1924 (Lehman 1928; Phillips 1999). FLS is an important foliar disease of soybean although symptoms can appear on stems, pods, and seeds. There has been no report of the alternative host in other crops or weeds (Mian et al. 2008). Initial symptom appears as small, light brown circular spot which is later surrounded by darkish brown to reddish circle. (Dashiell and Akem 1991). As the leaves are covered with 50% lesions, leaves start to blight wither and finally falls prematurely. On the lower surface of leaves, the central spot of lesions is somewhat grayish because of conidia produced on conidiophores. Conidia are a primary and secondary source of inoculum and are produced in infected leaves, stems, and pods. Warm temperature and frequent rainfall are suitable factors for severe disease, and fully expanded leaves are more resistant with small lesions as compared to younger leaves (Phillips 1999).

The United States is the leading producer of soybean in the world. According to the food and agriculture organization (FAO), the US produced 108 million metric tons of soybeans, second only to corn in 2014 (http://faostat3. fao. org/). FLS is an important disease in most of the soybean growing countries in the world and the main factors hindering the yield includes a reduction in photosynthetic area and premature defoliation of leaves (Mian et al. 2008; Wrather et al. 2010). In the US, FLS is significantly present in Southern warm

and humid regions (Mian et al. 2008; Yang et al. 2001). Now, *C. sojina* is also important to Northern states as the disease was reported in Iowa in 1999, Wisconsin in 2000 (Mengistu et al. 2002) and Ohio in 2006 (Cruz and Dorrance 2009). The damage caused by FLS depends on soybean cultivars and locations, and yield loss has been reported from 10% to more than 60% (Dashiell and Akem 1991; Hartwig and Edwards Jr 1990; Laviolette et al. 1970; Mian et al. 1998).

FLS is a polycyclic disease and the disease remains active throughout the growing season (Kim et al. 2013; Laviolette et al. 1970). Dispersal of conidia to some distance is favored by the wind and water splashes (Laviolette et al. 1970). Mycelium of *C. sojina* can overwinter and a report suggests potential survival of the pathogen in the plant debris for two years (Zhang and Bradley 2014). There are several FLS control methods including cultural practices, use of fungicides and genetic resistance. Primarily, genetic resistance is a most effective measure to control FLS. Till now, three resistant genes *Rcs* (Resistant to *C. sojina*), have been deployed: *Rcs1* (Athow and Probst 1952), *Rcs2* (Athow et al. 1962) and *Rcs3* (Phillips and Boerma 1982). The *Rcs3* gene confers resistant against race 5 and all known races of *C. sojina* present in the USA. (Mengistu et al. 2012; Phillips and Boerma 1982). Similarly, crop rotation for two years has been suggested to skip viable inoculum and prevent disease severity in the field (Grau et al. 2004; Zhang and Bradley 2014). Further, use of pathogen-free seeds and necessary application of fungicides before flowering to early pod stage have been practiced to decrease disease severity (Grau et al. 2004). Meanwhile, because of change in the pathogen, it has been proven that resistant gene

can confer resistance for a certain period and there can be selection against QoI fungicides too (Athow and Probst 1952; Athow et al. 1962; Zeng et al. 2015). There has already been a report of field isolates resistant to QoI fungicides in Tennessee (Zhang et al. 2012). Control measures like use of fungicides and planting of resistant cultivars force pathogens to select against selection pressure.

Studies of *C. sojina* using several approaches indicate diversity among isolates. Because of the lack of universally accepted soybean differentials, it's hard to characterize and compare *C. sojina* isolates. Grau et. al. (2004) have reported 12 races of *C . sojina* in the US, 22 races in Brazil and 14 races in China. A new set of 12 soybean differentials and 11 races have been proposed based on the reaction of isolates collected from the USA, Brazil, and China (Mian et al. 2008). However, the reaction of 50 isolates from Ohio on the same 12 soybean differentials produced 20 different races (Cruz and Dorrance 2009). There has been a handful of research to characterize *C. sojina* based on molecular markers. One study includes AFLP based assessment of 62 isolates from Brazil, China, Nigeria and the United States, which showed a significant amount of genetic diversity among isolates, although genotypes did not cluster based on origin. (Bradley et al. 2012). Recently, a study of 132 isolates from Arkansas with simple sequence repeat (SSR) has shown the chances of sexual reproduction and high genetic diversity in *C. sojina* (Kim et al. 2013).

The main objectives of this study were to access: genetic diversity by developing and using novel SNP markers and distribution of QoI resistant and sensitive isolates from Jackson and Milan, TN.

Sample collection, Single-lesion Isolation, and DNA extraction

In 2015, soybean leaves exhibiting typical symptoms of infection with FLS were collected from research plots at two locations in Tennessee (Milan and Jackson). In total, 437 isolates, 203 from Jackson and 234 from Milan, were collected from eight fungicides treated and non-treated Maturity group III soybean cultivars (Table 1). Cultivars were planted in 4 rows (30-inch row spacing), 30 ft long plots in a randomized complete block design with four replications. Plots were split, two rows were not treated, and two rows were treated at R3 growth stage (beginning pod) with Quadris Top SB at 8 fl oz/a (Azoxystrobin and Difenoconazole, Syngenta Corp., Basel, Switzerland). A single isolate of *C. sojina* was obtained from a single lesion from each leaf. Sporulation was induced by incubating leaves in a plastic bag with moist towels at room temperature. Spores were harvested with a flame-sterilized needle using a dissecting microscope and 8-10 spores transferred to RA-V8 agar media (rifampicin 25 ppm, ampicillin 100 ppm, 160 mL unfiltered V8 juice, 3 gm calcium carbonate and 840 mL water). Observations were made daily and contaminated sectors removed. After seven days, single-lesion isolates of *C. sojina* were transferred to a new V8 agar media. In addition, a set of 40 isolates from 10 different states, collected before 2015, were included in this study (Table 2).

Table 1. Soybean cultivars and number of *Cercospora sojina* isolates recovered from treated and non-treated cultivars.

Cultiv	Cultivars	Jackson	Milan	Tota

ar ID		Treated	Non-treated	Treated	Non-treated	l
C1	VAR Armor 37-R33 RR2	17	11	21	4	53
C2	VAR Asgrow AG3832 GENRR2Y	7	15	20	14	56
C3	VAR Beck’s 393R4	0	0	0	3	3
C4	VAR Croplan R2C 3984	19	13	11	14	57
C5	VAR Mycogen 5N393R2 RR2 g	12	20	17	28	77
C6	VAR Terral	10	15	13	16	54

	REV 39A35					
C7	VAR USG 73P93R	22	6	13	21	62
C8	VAR Warren Seed 3780 R2Y It	14	22	13	26	75

Table 2. Number of *Cercospora sojina* isolates collected from Jackson (JTN) and Milan (MTN), Tennessee in 2015 and historical isolates from various states in previous years.

Location	No. of Samples	Year
JTN	203	2015
MTN	234	2015
AL	5	2006
AR	5	2006
FL	1	2006
GA	4	2006

IA	1	2006
IL	2	2006/09
LA	1	2006
MS	6	2006
SC	2	2006/2009
TN	12	2007
WI	1	2006

Note: JTN (Jackson) and MTN (Milan) collection in 2015 in Tennessee. TN is a historical collection.

For DNA extraction, the single-lesion isolates were grown in 24-well deep well plates (Fisher Scientific) with 1 mL RA-V8 liquid broth (same as above, minus the agar) per well. DNA was extracted as described by Lamour and Finley (2006). Briefly, this includes harvesting mycelium from the broth cultures into a 96-well 2 mL deep well plate pre-loaded with 3-5 sterilized 3 mm glass beads. The plates are freeze dried and the dried mycelium powdered using a Mixer-Mill bead beating device (Qiagen). The powdered mycelium was then lysed and a standard glass fiber spin-column DNA extraction completed. The resulting genomic DNA was visualized on a 1% gel and quantified using a Qubit device.

SNP marker discovery and targeted-sequencing based genotyping

Whole genome sequencing was accomplished for three FLS isolates from a historical collection originally compiled by Dr. Dan Philips, UGA: FLS11 (CS10117) recovered from Milan, Tennessee in 2010, FLS19 (TN10) from the Georgia Experiment Station, and FLS21 (TN85) which was recovered from Mississippi. Genomic DNA was extracted from freeze-dried and powdered mycelium using a standard phenol-chloroform approach and the resulting DNA was submitted to the Beijing Genomics Institute in China for 2×100 paired-end sequencing on an Illumina HiSeq2000 device. *De novo* assembly, read mapping and SNP discovery was accomplished with CLC Genomics Workbench 7 (Qiagen). As there was no public reference genome available at the time, FLS21 was *de novo* assembled using the default settings in CLC and the resulting contigs used as a reference genome. All open reading frames (ORFs) longer than 300 amino acids were predicted using CLC and annotated onto the FLS21 contigs. The raw reads from FLS11 and FLS19 were then mapped to the draft reference (separately), and putative single nucleotide variants (SNVs) identified at sites with at least 20X coverage and an alternate allele frequency greater than 90%.

A subset of the SNVs was chosen from the largest contigs for further genotyping using a targeted sequencing approach. Custom Perl scripts were used to extract the flanking sequences for the panel of SNPs and primers were designed using BatchPrimer3 v1. 0 (http://probes. pw. usda. gov/batchprimer3/) to amplify targets between 80 and 120bp in length. Primers for 50 SNPs including mitochondrial QoI resistant locus are

summarized in Table 3. Primer sequences and genomic DNA were sent to Floodlight Genomics (Knoxville, TN) for processing as part of a non-profit Educational and Research Outreach Program (EROP) that provides targeted-sequencing services at cost for academic researchers. Floodlight Genomics uses an optimized Hi-Plex approach to amplify targets in multiplex PCR reactions and then sequences the resulting sample-specific amplicons on either an Illumina or Ion NGS device. Resulting sample-specific sequences were mapped to the reference contigs and genotypes assigned for loci with at least 6X coverage.

QoI resistant locus genotyping

A single nucleotide polymorphism (G/C) in the Cytochrome b gene of the *C. sojina* mitochondrial genome has been shown to confer resistance to QoI fungicides. A custom TaqMan SNP genotyping assay will be designed using the online design tools from Applied Biosystems (Thermo Scientific) and include the forward primer GGGTTATGTTTTACCTTACGGACAAATG and reverse primer GTCCTACTCATGGTATTGCACTCA and two probes to discriminate resistant and sensitive isolates: ACTGTGGCA G CTCATAA with VIC for the “ C” resistance allele and ACTGTGGCA C CTCATAA with FAM for the “ G” sensitive allele (Zeng et al. 2015). Quantitative PCR (qPCR) will be accomplished based on manufacturer instruction using the QuantStudio 6 Flex Real-time PCR System (Thermo Fisher Scientific Inc.).

Mating types determination

A previously described multiplex PCR assay will be used to assign mating type (MAT1-1-1 or MAT1-2) to a subset of the isolates that had unique multi-locus SNP genotypes (Kim et al. 2013). The *MAT1-1-1* locus will be amplified

with CsMat1f (5′ TGAGGACATGGCCACCCAAATA) and CsMat1r (5′ AAGAGCCCTGTCAAGTGTCAGT) and the *Mat1-2* locus will be amplified with CsMat2f (5′ TGTTGTAGAGCTCGTTGTTCGCA) and CsMat2r (5′ TCAGACCTTATGAGCTTGAAAGTGCT) primers (Kim et al. 2013). The assay will be included with the ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′ TCCTCCGCTTATTGATATGC) primers as an internal control to amplify the internal transcribed spacer (ITS) region (White et al. 1990). The resulting PCR products will be visualized under UV light on 2% agarose gel stained with GelRed (Phenix Research Products) and scored based on fragment size of *MAT1-1-1* (405 bp) and *Mat1-2* (358 bp).

Data Analysis

SNP loci for each sample will be combined to form a multi-locus SNP genotypes and samples with identical genotype (clonal lineages) will be clone corrected. To assess population structure among the two locations (and in relation to the historical isolates), Bayesian clustering will be accomplished using Structure 2. 3. 4 (Pritchard et al. 2000). Structure Harvester (Earl 2012) will be used to find the most probable value of K from the results obtained from Structure analysis. Principle coordinate analysis, AMOVA, Nei pairwise genetic distance, Nei pairwise genetic identity and genetic indices will be analyzed with GENALEX (Peakall and Smouse 2006). Phylogenetic clustering of the unique genotypes will be accomplished using Mega 6. 06 (Tamura et al. 2013). Minimum spanning networks (Bandelt et al. 1999) will be constructed with PopART (http://popart. otago. ac. nz/).

Expected Results

Novel SNP markers will be developed and assayed in *C. sojina* isolates. Population study will help to determine if the isolates from two locations are sub-grouped. The genetic study will also accesses genetic diversity present within and among populations.

Molecular identification of mutated cytochrome b site will help to determine the distribution of resistant isolates and contribute to compare resistant isolates in fields between two different time periods. Study of two different mating types in population will help to predict sexual reproduction.

References

Athow K and Probst AH. 1952. The inheritance of resistance to frog-eye leaf spot of Soybeans. Phytopathology 42(12): 660-662 pp.

Athow KL, Probst AH, Kurtzman CP and Laviolette FA. 1962. A newly identified physiological race of *Cercospora sojina* on soybean. Phytopathology 52(7): 712-714 pp.

Bandelt H-J, Forster P and Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular biology and evolution 16(1): 37-48.

Bradley C, Wood A, Zhang G, Murray J, Phillips D and Ming R. 2012. Genetic diversity of *Cercospora sojina* revealed by amplified fragment length polymorphism markers. Canadian Journal of Plant Pathology 34(3): 410-416.

Cruz C and Dorrance A. 2009. Characterization and survival of *Cercospora sojina* in Ohio. Plant Health Progress doi 10.

Dashiell K and Akem C. 1991. Yield losses in soybeans from frogeye leaf spot caused by *Cercospora sojina* . Crop Protection 10(6): 465-468.

Earl DA. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation genetics resources 4(2): 359-361.

Grau CR, Dorrance AE, Bond J and Russin JS. 2004. Fungal diseases. Soybeans: Improvement, production, and uses(soybeansimprove): 679-763.

Hartwig E and Edwards Jr C. 1990. The uniform soybean tests, southern region, 1989. USDA Mimeographed Rep. US Gov. Print. Office, Washington, DC.

Kim H, Newell AD, Cota-Sieckmeyer RG, Rupe JC, Fakhoury AM and Bluhm BH. 2013. Mating-type distribution and genetic diversity of *Cercospora sojina* populations on soybean from Arkansas: Evidence for potential sexual reproduction. Phytopathology 103(10): 1045-1051.

Laviolette F, Athow K, Probst A, Wilcox J and Abney T. 1970. Effect of bacterial pustule and frogeye leafspot on yield of Clark soybean. Crop science 10(4): 418-419.

Lehman S. 1928. Frog-eye leaf spot of Soy Bean caused by *Cerco-spora diazu* Miara. Journal of Agricultural Research 36(9): 811-833.

Mengistu A, Bond J, Mian R, Nelson R, Shannon G and Wrather A. 2012. Resistance to Frogeye Leaf Spot in selected soybean accessions in MG I through MG VI. Plant Health Progress 10.

Mengistu A, Kurtzweil NC and Grau CR. 2002. First report of Frogeye Leaf Spot (*Cercospora sojina*) in Wisconsin. Plant Disease 86(11): 1272-1272.

Mian M, Boerma H, Phillips D, Kenty M, Shannon G, Shipe E, Blount AS and Weaver D. 1998. Performance of frogeye leaf spot-resistant and-susceptible near-isolines of soybean. Plant disease 82(9): 1017-1021.

Mian M, Missaoui A, Walker D, Phillips D and Boerma H. 2008. Frogeye Leaf Spot of Soybean: A review and proposed race designations for isolates of Hara. Crop science 48(1): 14-24.

Peakall R and Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular ecology notes 6(1): 288-295.

Phillips D. 1999. Frogeye leaf spot. Compendium of soybean diseases, 4th ed. American Phytopathological Society Press, St. Paul, MN: 20-21.

Phillips D and Boerma H. 1982. Two genes for resistance to race 5 of *Cercospora sojina* in soybeans. Phytopathology 72(7): 764-766.

Pritchard JK, Stephens M and Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155(2): 945-959.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6. 0. Molecular biology and evolution: mst197.

White TJ, Bruns T, Lee S and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18(1): 315-322.

Wrather A, Shannon G, Balardin R, Carregal L, Escobar R, Gupta G, Ma Z, Morel W, Ploper D and Tenuta A. 2010. Effect of diseases on soybean yield in the top eight producing countries in 2006. Plant Health Progress doi 10: 2008-2013.

Yang X, Uphoff M and Sanogo S. 2001. Outbreaks of soybean frogeye leaf spot in Iowa. Plant Disease 85(4): 443-443.

Zeng F, Arnao E, Zhang G, Olaya G, Wullschleger J, Sierotzki H, Ming R, Bluhm B, Bond J and Fakhoury A. 2015. Characterization of quinone outside inhibitor fungicide resistance in *Cercospora sojina* and development of diagnostic tools for its identification. Plant Disease 99(4): 544-550.

Zhang G and Bradley CA. 2014. Survival of *Cercospora sojina* on soybean leaf debris in Illinois. Plant Health Prog 10.

Zhang G, Newman M and Bradley C. 2012. First report of the soybean frogeye leaf spot fungus (*Cercospora sojina*) resistant to quinone outside inhibitor fungicides in North America. Plant Disease 96(5): 767-767.