METABOLOMIC AND PROTEOMIC PROFILING FOR UNDERSTANDING SUGAR BEET RESISTANCE TO *RHIZOCTONIA SOLANI* AG 2-2 IIIB.

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Rhizoctonia root rot (caused by the fungus *Rhizoctonia solani* Kühn) continues to be a problem in most sugar beet-growing areas in the United States, and is a growing problem worldwide (*1, 2*). Little is understood about how root pathogens such as *R. solani* interact with sugar beet and their mechanisms for resistance. Identification of novel sources of germplasm with broad-spectrum tolerance would greatly benefit the sugar beet community but screening can be tedious and long within traditional breeding programs. While significant progress has been made in breeding for resistance to Rhizoctonia root and crown rot (*3*) little is known about the specific genes and mechanisms that condition host resistance. Resistance to *R. solani* is believed to be polygenic, involving at least two loci, with two or three alleles, and an unknown number of modifying genes contributing to resistance (*4*).

Quantitative resistance mechanisms generally involve both metabolites and proteins (*5*). In order to characterize and better understand these mechanisms, functional genomics including transcriptomics, proteomics, and metabolomics, can reveal the potential biochemical pathways that serve as a basis of resistance (*6*). While functional genomics has traditionally been approached as a unidirectional flow of information from gene to transcript to protein, we now know that enzymes can affect metabolic pathways leading to phenotypic changes in the host and this traditional "linear" thinking is no longer true (*7*). In reality these cellular processes are intimately networked and contain many overlapping feedback-loops with protein complexes interacting with many metabolites in response to pathogen attack. The metabolome is the quantitative complement of all the low-molecular weight molecules present in the plant cell and is believed to be complementary to transcriptomic and proteomic studies to better characterize the defense response (*7*).

While proteomics has been used to characterize protein changes that occur during infection by the soilborne fungi *Fusarium oxysporum* and the fungi-transmitted virus *Beet Necrotic Yellow Vein Virus* (BNYVV) (*8-10*) resistance to each particular pathogen, such as the necrotrophic *R. solani,* needs to be studied separately to identify novel mechanisms for disease control. Metabolomic profiling has been previously used in sugar beet to complement proteomic studies to characterize the impacts of carbohydrate metabolism and TCA cycle pathways in response to iron deficiency (*11*). The objectives of this work were to profile the sugar beet metabolome in order to characterize the biochemistry that underlies the complex phenotype of Rhizoctonia root rot resistance by measuring the inherent metabolic variation occurring in a given system and use this information to further narrow sample type (i.e. tissue, timepoint, variety) for future functional genomic studies. While we previously characterized the metabolites that are differentially expressed in sugar beet during early interactions with *R. solani* AG 2-2 IIIB, we needed to characterize these same interactions at later time points during the infection cycle to represent more effectively how *R. solani* causes disease in the field.

Materials and Methods

Plant treatment(s) Sugar beet lines FC709-2 (resistant) and FC901 (susceptible) were grown in a greenhouse until 10 weeks after sowing. Uninoculated/sterile barley grains or *R. solani* AG2-2 IIB (isolate, R-9) infested barley grains were used to inoculate three pots each containing 3 plants for each variety by placing ~1/8 tsp. (about 0.50 - 0.55 g) of ground barley in the soil next to tap root (about 1-2 cm deep). Soil was kept moist, but not sodden by daily watering and placed in a greenhouse at 28°C. Plant tissue was collected from each pot at 0, 1, 3, 5, and 7 days after inoculation (Objective 1) and 0, 7, 14, and 21 dai (Objective 2) with root and leaf tissue kept separate. Experiments were biologically replicated two to three times.

Metabolite extraction For metabolite extraction, at each time point, leaf and root tissue were collected and treated separately. Leaf tissue was ground in liquid nitrogen, while root tissue was washed in distilled water to remove soil, cut into small slices, and frozen at -80°C. Metabolites were extracted using an 80% methanol extraction method (*6*) where we place 20mg of ground lyophilized tissue into a 1.5mL microcentrifuge and then add 1mL of 80% methanol/water (80v:20v:v) and vortex at room temperature for 2 hours. After vortexing, the samples were incubated at -20°C for 20 minutes and then centrifuged at 16,000rpm for 20 minutes at 4°C. Metabolites were removed as the supernatant fraction after centrifugation and placed into a new 1.5mL microcentrifuge tube which was kept at -80°C until mass spectrometry could be performed.

Metabolomic analysis Metabolite extract was injected onto a Waters Acquity UPLC system and separated using a Waters Acquity UPLC T3 column (1.8 lM, 1.0 9 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (acetonitrile, 0.1% formic acid). Injections were made in 100% A, held at 100% A for 1 min and following a linear gradient to 95% B in 12 min, held at 95% B for 3 min, returned to starting conditions over 0.05 min and allowed to re-equilibrate for 3.95 min, with a 200 lL/min flow rate. The column and samples were held at 50°C and 5°C, respectively. Column eluent is infused into a Waters Xevo G2 Q-TOF-MS with an electrospray source in positive mode, scanning 50–1200 m/z at 0.2 s per scan, alternating between MS (6 V collision energy) and MSE mode (15–30 V ramp). Calibration is performed using sodium formate with 1 ppm mass accuracy. The capillary voltage is held at 2200 V, source temp at 150 °C and nitrogen desolvation temp at 350 °C with a flow rate of 800 L/h. MS files were converted to netCDF format and processed with XCMS for peak detection, retention time alignment and feature grouping on both low and high collision energy channels. The feature based XCMS data are then subjected to RAMClustR (*12*). Spectra are then searched against in-house, NISTv14, Metlin and Massbank databases for annotations. Metabolite isomers are inferred by identical idMS and idMS/MS spectra at multiple retention times, and glycerolipid positional isomers classified as previously described (*6*).

Results and Discussion

Metabolomic profiling has been completed for all experiments associated with Objective 1. Here we saw very little influence of inoculation with *R. solani* at the earliest time points, but did see a slight change of metabolite profiles by the last day of the study (7dai) which could possibly be explained by infection of the pathogen (mock vs. inoculated treatments). It has been reported that during the *R. solani* infection process the pathogen must 1) first grow towards the plant, 2) grow over the plant, 3) attaches to the plant and then 4) produce the necessary infection structures which are then used to 5) penetrate the host and 6) finally colonize host tissues. The time and extent of this process depends on the specific isolate of *R. solani* as well as the host that it is infecting and therefore full colonization of the cortex has been reported to take up to 3-4 days after infection was initiated. More than 900 compounds were detected in either sugar beet roots or leaves, of which 143 were annotated. There was a clear distinction between tissue type and genotype, and more subtle changes in response to inoculation. Several biochemical pathways appear to be involved during susceptible and resistant interactions with *R. solani*, and indicate a complex role of primary and secondary metabolites in sugar beet during fungal interactions.

Greenhouse experiments for the characterization of the late stage (0, 7, 14, and 21dai) interactions of sugar beet during *R. solani* infection and metabolite extractions have been completed on all samples. Likewise the mass spectrometry has also been completed on the metabolite extractions from all samples. Data analysis and annotation of specific metabolites is currently in process. Preliminary results support previous data seen for sugar beet interactions during very early time points (0 and 7dai) in that metabolite expression is very different based on sugar beet germplasm and that primary metabolites are more prevalent than secondary

metabolites in roots. Additionally the metabolite profiles in sugar beet are impacted by infection by *R. solani* starting by 7dai. Analysis of metabolites expression is still in progress for later time points (14 and 21dai) and annotation of specific metabolites is still in progress for all treatments.

An important product of the findings of the first dataset from Objective 1 is that we now have an internal database of annotated metabolites in sugar beet that can then be used with the mass spectrometry data that we obtain from Objective 2 extractions. Because the metabolite structures should be the same, we are able to identify and annotate individual metabolites in a more timely fashion and with the overlap in time points between the two datasets be able to expand on the data set of metabolites found in sugar beet for use in future experiments.

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