DIFFERENTIATING RZ-1 AND RZ-2 RESISTANT REACTIONS TO *BEET NECROTIC YELLOW VEIN VIRUS* IN SUGARBEET

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Approximately four years ago, the Wintermantel Lab, in collaboration with the Sugarbeet Research Unit in Ft. Collins and the USDA-ARS Eastern Regional Research Center Core Lab, began identifying changes in the sugarbeet proteome (proteins produced by sugarbeet) between resistant and susceptible sugarbeet during a healthy (uninoculated) interaction and when infected by *Beet necrotic yellow vein virus* (BNYVV). This work was published in Physiological and Molecular Plant Pathology (Larson et al., 2008) and demonstrated that a relatively small number of changes in sugarbeet protein expression were associated with BNYVV infection as well as resistance.

Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV), is one of the most economically important diseases affecting sugarbeet, and is widely distributed in most sugarbeet growing areas of the world. Fields remain infested with BNYVV indefinitely in *P. betae* cystosori that remain dormant up to 25 years. Therefore rotation to non-host crops or lengthening rotations is ineffective at reducing disease incidence, and the only viable means of control has been natural host-plant resistance. Following the introduction of *Rz*1 varieties of sugarbeet in the 1990s, new pathotypes that break resistance have appeared. Additional sources of resistance have been identified and they hold promise. However the different sources of *Rz*-mediated resistance, map to different chromosomal positions and appear to have different underlying mechanisms which are largely unknown (Scholten *et al.*, 1997; 1999; Gidner *et al*., 2005). Furthermore, several minor genes other than primary *Rz* genes may contribute to more enhanced resistance (Gidner *et al*., 2005), which to date are not known. Until the epidemiology behind the spread of resistance-breaking isolates and the various mechanisms of resistance are understood, alternative disease control methods and additional sources of resistance will be required to control this pathogen. This project complements and builds upon work perfomed by Bob Lewellen in Salinas on rhizomania resistance, and by Hsing-Yeh Liu in Salinas and Charlie Rush at Texas A&M on resistance-breaking variants of BNYVV, and David Gilmer and Mark Varrelman on BNYVV-sugarbeet interactions in Europe.

The project will provide knowledge of the basis for why BNYVV pathotype IV (the resistance-breaking pathotype from California's Imperial Valley) overcomes resistance in sugarbeet containing the *Rz*1 resistance gene, but is unable to infect and cause disease on beets containing the *Rz*2 resistance gene. This will build on the information generated through our previous study, which identified protein interactions responsible for infection of sugarbeet by BNYVV pathotype A (the form common throughout the US) and development of rhizomania disease in sugarbeet, as well as differences that occur in these reactions between resistant and susceptible sugarbeet varieties. In addition, these studies may lead to methods to prolong the longevity of *Rz* resistance sources by understanding the fundamental mechanisms that cause resistance to break down. We intend to build on the information generated previously on BNYVV pathotype A (traditional form of BNYVV in US that is controlled by *Rz*1 resistance), by examining differential expression with infection by the *Rz*1 resistance-breaking BNYVV-IV (Imperial Pathotype). Although resistance-breaking isolates have been identified from all American sugarbeet production regions, to date the *Rz*1 resistance gene has only been overcome in the Imperial Valley. These studies should allow us to gain a much clearer understanding of what changes occur in beet during BNYVV infection. We can compare infection of a susceptible beet (*rz*) with two different forms of resistance (*Rz*1, which is overcome by the Imperial Pathotype, and *Rz*2, which is resistant to the Imperial Pathotype). Comparing protein profiles should allow us to identify the anticipated minor changes that occur in resistant beet (*Rz*2), beet in which resistance is compromised (*Rz*1), and compare these to susceptible beet (*rz*1, *rz*2) and with expression profiles generated during our previous studies on Pathotype A.

OBJECTIVES

1. Grow near isogenic lines of sugarbeet containing either resistance (Rz1 or Rz2 genotype) or susceptibility (rz1 + rz2 genotype) under standardized growth chamber conditions, with and without infection by BNYVV-IV (resistance breaking pathotype), and extract total proteins from root tissue.
2. Analyze differential protein expression among the treatments listed in Objective 1, following protein separation using the NanoAcquity UPLC-2D System; and enter proteins into the *Beta vulgaris* Genome Initiative database.

MATERIALS AND METHODS

An initial test designed to identify and eliminate any unforeseen issues that could develop during the actual experimentation was successful, with all plants performing with expected growth habit and root symptoms of rhizomania (or not) as would be expected depending on whether the seed line was resistant (Rz2) or susceptible (Rz1 or rz1) to the Imperial Pathotype of BNYVV. Infection of seedlings by BNYVV was confirmed by ELISA, the serological testing method used universally by the sugarbeet industry to confirm BNYVV infections. Results of preliminary testing demonstrated protein extractions were performing effectively.

Sugarbeet varieties for protein analysis have nearly identical genetic background (near isogenic lines) essentially differing only for rhizomania resistance. Lines were provided through Material Transfer Agreement with KWS (Einbeck, Germany), since near isogenic lines from the previous study to which we are comparing newly generated information were also provided by KWS. The diploid beet varieties will each carry one of three genetic backgrounds in response to BNYVV-IV (Imperial Valley Res. Breaking Isolate [aka. Imperial Pathotype]): Susceptible (rz1,rz1; rz2,rz2), Resistant (rz1,rz1; Rz2,Rz2); and susceptible to the isolate used, but resistant to the more common pathotype A (Rz1,Rz1; rz2,rz2).

A biologically characterized source of BNYVV-IV was collected from the Hartnell College field where the Imperial Pathotype of BNYVV has been propagated adjacent to the USDA-ARS in Salinas, CA. The original source of this isolate was from the field, Rockwood 158, Imperial County, CA (Liu et al., 2005). Infested soil samples were mixed in equal parts with autoclaved builders' sand to facilitate ease of root removal at harvest as in previous studies. Soil was placed in new 280 ml Styrofoam cups with holes punched in the bottom for drainage and placed in sterilized plastic saucers spaced in growth chambers to avoid contamination by splashing water between cups. Growth chambers were washed in 10% sodium hypochlorite prior to use to remove any possible contamination. Approximately 100 sugar beet seeds of each variety were layered on top of each cup. Seeds within each cup were covered with sand to a depth of about 1 cm, and the cups will be watered with gentle misting as needed to germinate sugar beet seedlings. Following germination, water was added to the saucers directly as needed to prevent wilting. All methods for planting and propagation are described in detail in Liu et al. (2005). Each treatment type was maintained individually in separate growth chambers set at 24 C (Conviron E15 Growth Chambers) to avoid any potential cross-contamination. Root samples (10 grams/treatment) were collected from individual plants and pooled at 3 weeks post seedling emergence, which corresponds to the early stages after initial viral infection and the beginning of symptom development, respectively. Roots were washed and lyophilized. Root samples from each pot were tested by ELISA to confirm the presence or absence of BNYVV using virus specific antiserum developed in the ARS virology laboratory in Salinas, CA using methods described by Wisler *et al* (1999; 2003). Samples with ELISA absorbance readings of at least 2 times the absorbance of healthy controls were considered infected, while absorbance reading less than 1.3 times the healthy controls were considered virus free. Confirmation of infection by the appropriate virus isolate was confirmed by infection phenotype on the roots based on known reaction of each genotype to the Imperial Isolate (BNYVV-IV).

Proteins from each representative sample were extracted from lyophilized (freeze-dried) root material (5 g) using the Plant Total Protein Extraction Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's directions, and quantified using standard methods (Bradford assay). Each plant variety, treatment combination is being extracted and analyzed in two independent replications.

Sugarbeet genotypes are described with respect to rhizomania resistance and soil types with and without *P. betae* carrying BNYVV in Table 1. Each treatment is listed with a code (A1 through C3), which is used for tracking treatments throughout the experiments.

Table 1. Sugarbeet genotypes and soil types used in experiments[1]

Sugarbeet Type	Healthy Soil (Hartnell)	BNVYY-IV Soil (Hartnell)	BNYVV-A Soil (Spence)
Near iso *Rz1rz2* (Rz1)	A1	A2	A3
Near iso *rz1Rz2* (Rz2)	B1	B2	B3
Near iso *rz1rz2* (susc.)	C1	C2	C3

[1] Codes A1 through C3 are used to track seed source and treatment combinations.

RESULTS AND DISCUSSION

At the beginning of this project, samples were to be analyzed by ultraperformance liquid chromatography and mass spectrometry at the USDA-ARS-ERRC in Wyndmoor, Pennsylvania; however, internal staffing and funding issues at ERRC resulted in the decision to shift analysis to Colorado State University using similar technology during the summer of 2011. Sample analysis began in summer 2011, and a number of proteins have been identified to date. Funds originally intended for hiring a bioinformatist are now being used to cover cost of processing through CSU. Additional funds to support this have been requested from multiple sugarbeet industry sources (Western Sugar Growers, CBGA [Calif. Growers], SBREB [MN/ND Growers], as well as BSDF) due to cost of processing**.**

Total protein extracts (200ug per sample) are being processed through SCX fractionation (strong cation exchange chromatography). The SCX fractionation separates each sample into smaller subsamples, reducing complexity. Following SCX fractionation, fractionated samples will be processed through reverse phase liquid chromatography and mass spectrometry (LC-MS-MS). Processing will be performed at the Colorado State University (CSU) Proteomics and Metabolomics Facility in Ft. Collins, CO. Peptide Spectra are then compiled using Mascot software and amino acid sequences are examined for identity using the NCBI 'all plant' database as well as the *Beta vulgaris* genome initiative (BvGI) database. This system eliminates the need to perform traditional two-dimensional separations using gel electrophoresis of fractionated proteins.

There were some issues with this experiment during protein separation and analysis at CSU this summer. A contaminating compound, triethylamine hydrochloride, was present in several of the samples during analysis that precluded protein identification during the initial experiment. The cause of the contaminant was investigated, and ultimately it was determined to have occurred during pre-separation processing. Although some of the samples were acceptable, it was necessary to repeat the experiment again to allow comparisons between relevant samples. To eliminate this issue, final protein preparation is now being performed at the CSU proteomics facility, to insure quality control at all stages of preparation and separation analysis. This may increase costs slightly, but should provide more accurate and efficient separation analysis.

A second planting was performed in November, with harvesting and lyophilization of proteins on Dec. 1, 2011. Our remaining initial samples are currently in the Queue at CSU for protein separation, and we are hopeful that some results will be available prior to the meeting in January, although as of the date of this report this seems unlikely. The set prepared in December (replication of initial set) should be processed later this winter following completion of set 1.

Cost Sharing:
This work is additionally supported by a number of sugar industry organizations including the Beet Sugar Development Foundation, the Western Sugar Cooperative-Grower Joint Research Committee, and the California Beet Growers Association which are providing funds along with ARS. Funding from multiple organizations facilitates our ability to complete the research.

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