DIFFERENTIATING *RZ-1* AND *RZ2* RESISTANCE REACTIONS TO *BEET NECROTIC YELLOW VEIN VIRUS* IN SUGARBEET

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Justification for Research:
Over the past few years, the Wintermantel Lab (ARS, Salinas, CA) and the ARS Sugarbeet Research Unit in Ft. Collins, CO have been studying 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 performed 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 is providing new information on physiological changes illustrated by protein expression variation among sugarbeet plants infected with a resistance-breaking BNYVV pathotype (BNYVV-IV), the resistance-breaking form of the virus from California's Imperial Valley, and the traditional A-pathotype (common throughout the US and world) and how this is influenced by the presence or absence of either the Rz1 or Rz2 resistance genes. This information builds on the information generated through our previous work, as well as parallel studies conducted in Europe, which identified protein changes associated with 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 biological pathways that become activated or altered during infection and resistance, including those that may be associate with break down of resistance. 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 on significant acreage in California's Imperial Valley, and even there spread has been limited. These studies should allow us to gain a much clearer understanding of what changes occur in beet during BNYVV infection. We compare infection of a susceptible beet (*rz*) with two different forms of resistance (*Rz*1, which is overcome by the Imperial Pathotype (BNYVV-IV), and *Rz*2, which is resistant to the BNYVV-IV). Comparing protein profiles is leading to identification of minor changes that occur in resistant beet (*Rz*2), beet in which resistance is compromised (*Rz*1), how protein expression differs from that in susceptible beets (*rz*1, *rz*2), and with expression profiles generated during previous studies on Pathotype A in our laboratories and those of other research groups.

Objective for 2011:
Complete protein analysis and evaluate differential protein expression among the treatments listed in Table 1, following protein separation using the liquid chromatography and mass spectrometry at CSU; and enter proteins into the *Beta vulgaris* Genome Initiative database.

Progress on Objectives:
Three independent growth chamber experiments were completed sequentially using the same growth chambers and growth parameters for all plants in all experiments to eliminate variability to the greatest extent possible.

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 and there was a benefit to using the same genetic material for comparative purposes. The diploid beet varieties each carried one of three genetic backgrounds in response to BNYVV: susceptible (rz1,rz1; rz2,rz2); resistant (Rz1 gene) to the widely prevalent BNYVV-A pathotype (Rz1,Rz1; rz2,rz2) but susceptible to Rz-1 resistance breaking strains of BNYVV; and resistant to both traditional BNYVV-A and current resistance breaking strains such as the Imperial Valley, CA isolate (BNYVV-IV) (rz1,rz1; Rz2,Rz2).
A biologically characterized source of resistance-breaking 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). All experiments were run sequentially and replicated three times in growth chambers.

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 (A2 through C3), which is used for tracking treatments throughout the experiments.

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

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

[1] Codes A2 through C3 are used to track seed source and treatment combinations.

Proteins from each representative sample were extracted from lyophilized (freeze-dried) plant material described above (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).

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 a different type of analysis 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 were used to cover cost of processing through CSU. Funds to support various aspects of this research have been provided by multiple sugarbeet industry sources (SBREB, Western Sugar Growers, California Beet Growers, as well as BSDF) due to cost of processing and labor.

Total protein extracts (200ug per sample) were processed at the Colorado State University (CSU) Proteomics and Metabolomics Facility in Ft. Collins, CO. All three replications were processed together at CSU to reduce variability. Peptide Spectra were then compiled using Mascot software and amino acid sequences examined for identity using the *Amaranthaceae* database because it was the most complete proteome database containing protein information related to sugarbeet. The sugarbeet public database, *Beta vulgaris* Genome Initiative (BVGI), did not have significant protein, but rather mRNA information and therefore was not appropriate for these comparisons. The NCBI 'all green plant' database is being used as a cross-reference source, but contains too many redundancies to facilitate efficient direct comparisons.

Mass Spectrometry Analysis
Peptides were purified and concentrated using an on-line enrichment column followed by chromatographic separation on a reverse phase nanospray column using a 90 minute linear gradient. Peptides were eluted directly into the mass spectrometer (Thermo Scientific LTQ linear ion trap). Compound lists of the resulting spectra were generated using Xcalibur 2.2 software (Thermo Scientific). Spectra obtained through mass spectrometry (MS/MS) were searched against the mouse Uniprot *Amaranthaceae* database (version 11/02/12) concatenated with reverse sequences for determination of the peptide FDR 1 (6,832 sequence entries) using both the Mascot database search engine (version 2.3) and Sorcerer™-SEQUEST®.

Search results for each independently analyzed sample were imported and combined using Scaffold software 3 (Version 3.6.4, Proteome Software, Portland, OR). Proteins containing shared peptides were grouped by Scaffold (Proteome Software, Portland, OR) to satisfy the laws of parsimony. Manual validation of MS/MS spectra was performed for all protein identifications above the probability thresholds that were based on only two unique peptides. Results identified nearly 500 proteins exhibiting variability. Those exhibiting clearly significant differences between traditional and resistance-breaking BNYVV strains within each sugarbeet genotype are shown below in **Table 2**; whereas significant differences in protein expression between resistance genotypes for each BNYVV strain are presented in **Table 3**. Many additional proteins were identified but were not listed as the differences were not statistically significant. However, some of these may be of interest to clarify pathways that may be altered by supporting other proteins identified as significantly different between treatments. Most of the statistically significant protein differences are associated with photosynthetic pathways, supporting the effect of BNYVV infection on foliar yellowing in the field. Others are involved in pathogen defense that are most intriguing. Of particular interest is a Beta-1,3-Glucanase, which is a protein that has been shown to be a likely pathogen response protein in corn with antifungal activity. Additionally, this protein may be correlated with ability of some viruses to induce symptoms and move systemically in infected plants. Another interesting protein is Glutamine synthetase, a protein involved in nitrogen regulation in plants. We suspect this protein may be involved in general BNYVV infection or *P. betae* pathogenesis. Other proteins will likely be of interest as well, but more in-depth interpretation of the data will be needed for clarification, and this will take some time.

In addition to clarification of protein expression and physiological pathways affected by infection and resistance, we plan to submit identified proteins to the BVGI database later this year, to facilitate future studies on sugarbeet proteins. We anticipate submission of at least one manuscript describing information generated by this project within the year, at which point more detailed information will be made available to the sugarbeet industry.

Table 2. Proteins exhibiting significantly different expression levels between traditional (BNYVV-A) or resistance breaking (BNYVV-IV) virus isolates within an individual resistance genotype (Rz1, Rz2, or susceptible).

A2 (Resistance-breaking BNYVV-IV) vs A3 (traditional BNYVV-A) in Rz1 Beet

Photosystem I iron-sulfur center OS=Spinacia oleracea

S-adenosylmethionine synthase 1 OS=Beta vulgaris

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Ofaiston monandrum

Photosystem II CP47 chlorophyll apoprotein OS=Spinacia oleracea

Peroxisomal (S)-2-hydroxy-acid oxidase OS=Spinacia oleracea

Adenosylhomocysteinase OS=Beta vulgaris

Glutamine synthetase OS=Spinacia oleracea

Phosphoribulokinase, chloroplastic OS=Spinacia oleracea

B2 (Resistance-breaking BNYVV-IV) vs B3 (traditional BNYVV-A) in Rz2 Beet

Cytochrome b559 subunit alpha OS=Beta vulgaris

Fructose-bisphosphate aldolase, chloroplastic OS=Spinacia oleracea

Ribulose bisphosphate carboxylase large chain OS=Atriplex rosea

Actin 1 OS=Celosia argentea

Photosystem I iron-sulfur center OS=Spinacia oleracea

33 kDa protein of the oxygen-evolving complex OS=Salicornia europaea

Glucan endo-1,3-beta-D-glucosidase OS=Beta vulgaris subsp. vulgaris

S-adenosylmethionine synthase 1 OS=Beta vulgaris

Photosystem II CP47 chlorophyll apoprotein OS=Spinacia oleracea

Phosphoglycerate kinase, chloroplastic (Fragment) OS=Spinacia oleracea

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Ofaiston monandrum

C2 (Resistance-breaking BNYVV-IV) vs C3 (traditional BNYVV-A) in susceptible rz1/rz2 Beet

Adenosylhomocysteinase OS=Beta vulgaris

Malate dehydrogenase, cytoplasmic OS=Beta vulgaris

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Pachycornia triandra

Glutamine synthetase OS=Spinacia oleracea

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Sclerostegia moniliformis

Phosphoribulokinase, chloroplastic OS=Spinacia oleracea

Fructose-bisphosphate aldolase, chloroplastic OS=Spinacia oleracea

ATP synthase subunit alpha, chloroplastic OS=Spinacia oleracea

Cytochrome b559 subunit alpha OS=Beta vulgaris

Table 3. Proteins exhibiting significantly different expression levels between resistant and susceptible genotype sugarbeet inoculated with either traditional (BNYVV-A) or resistance breaking (BNYVV-IV) virus isolates.

C2 vs A2 (Susceptible rz1/rz2 vs. "Susc." Rz1/rz2 inoculated with RB-BNYVV-IV)

33 kDa protein of the oxygen-evolving complex OS=Salicornia europaea

Fructose-bisphosphate aldolase, chloroplastic OS=Spinacia oleracea

Photosystem II CP47 chlorophyll apoprotein OS=Spinacia oleracea

Photosystem I iron-sulfur center OS=Spinacia oleracea

Chlorophyll a /b binding protein OS=Beta vulgaris

Glyceraldehyde-3-phosphate dehydrogenase OS=Atriplex nummularia

Glyceraldehyde-3-phosphate dehydogenase OS=Beta vulgaris
Transketolase, chloroplastic OS=Spinacia oleracea

Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic OS=Spinacia oleracea

Fructose-bisphosphate aldolase, chloroplastic OS=Spinacia oleracea

Chlorophyll a /b binding protein OS=Beta vulgaris

Photosystem I iron-sulfur center OS=Spinacia oleracea
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic OS=Spinacia oleracea

C2 vs B2 (Susceptible rz1/rz2 vs. Resistant rz1/Rz2 inoculated with RB-BNYVV-IV)

ATP synthase subunit alpha, chloroplastic OS=Spinacia oleracea

Major chlorophyll a/b binding protein LHCb1.3 OS=Spinacia oleracea

Photosystem I reaction center subunit IV, chloroplastic OS=Spinacia oleracea

C3 vs B3 (Susceptible rz1/rz2 vs. Resistant rz1/Rz2 inoculated with BNYVV-A)

Photosystem I iron-sulfur center OS=Spinacia oleracea

Actin 1 OS=Celosia argentea

Cytochrome b559 subunit alpha OS=Beta vulgaris

Phosphoglycerate kinase, chloroplastic (Fragment) OS=Spinacia oleracea

Glyceraldehyde-3-phosphate dehydogenase OS=Beta vulgaris

Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic OS=Spinacia oleracea

Photosystem I iron-sulfur center OS=Spinacia oleracea

C3 vs A3 (Susceptible rz1/rz2 vs. Resistant Rz1/rz2 inoculated with BNYVV-A)

Cytochrome b559 subunit alpha OS=Beta vulgaris

Phosphoglycerate kinase, chloroplastic (Fragment) OS=Spinacia oleracea

Actin 1 OS=Celosia argentea

Glutamine synthetase OS=Spinacia oleracea

Ribulose bisphosphate carboxylase large chain OS=Beta vulgaris

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Sclerostegia moniliformis

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Pachycornia triandra

Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic OS=Spinacia oleracea

ClpC protease OS=Spinacia oleracea

Glyceraldehyde-3-phosphate dehydogenase OS=Beta vulgaris P

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) OS=Pachycornia triandra

Adenosylhomocysteinase OS=Beta vulgaris

Cost Sharing:

This work was supported by a number of sugar industry organizations including the Beet Sugar Development Foundation, the Sugar Beet Research and Education Board, the Western Sugar Cooperative-Grower Joint Research Committee, and the California Beet Growers Association which provided funds along with ARS. Funding from multiple organizations facilitated our ability to complete the research.

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