PROTEOME CHANGES IN SUGARBEET IN RESPONSE TO *BEET NECROTIC YELLOW VEIN VIRUS* INFECTION

W. M. Wintermantel[1]*, A. Gulati-Sakhuja[1], R. L. Larson[2], L. L. Hladky[1], A. Nunez[3] and A. Hill[4]
[1]USDA-ARS, 1636 East Alisal Street, Salinas, CA 93905, [2]Syngenta Seeds, 1020 Sugarmill Road, Longmont, CO 80501, [3]USDA-ARS-ERRC, 600 East Mermaid Lane, Wyndmoor, PA 19038 and [4]USDA-ARS, 1701 Centre Avenue, Fort Collins, CO 80526

Summary:

Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV), is a devastating viral pathogen of sugar beet.   There are limited sources of resistance against the virus and resistance-breaking isolates are becoming increasingly problematic worldwide. Developing more effective disease control strategies starts with gaining a better understanding of the basis for resistance and the mechanism of disease.   Multidimensional liquid chromatography was employed to examine proteins differentially expressed in nearly isogenic lines of sugar beet either resistant or susceptible to BNYVV infection. More than 1,000 protein peaks were reproducibly detected in the root extracts from each treatment. Differential protein expression in response to viral inoculation was determined by comparing healthy and BNYVV-challenged chromatogram protein profiles for each sugarbeet genotype. Protein expression was temporally regulated, and in total, 7.4 and 11% of the detected proteome was affected by BNYVV-challenge in the resistant and susceptible genotype, respectively. Sixty-five of the proteins induced or repressed by the virus were identified by tandem MALDI-TOF mass spectrometry and expression of key defense- and disease-related proteins was further verified using qualitative reverse transcriptase polymerase chain reaction.   The proteomic data suggests involvement of classic systemic resistance components in *Rz*1-mediated resistance and phytohormones in hairy root symptom development.

Introduction:

Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV; Tamada and Baba, 1973), is one of the most economically important diseases affecting sugar beet (*Beta vulgaris* L.), and is widely distributed in most sugar beet growing areas of the world. The disease is characterized by excessive growth of lateral roots and constriction of the taproot which reduces sugar yield. BNYVV is transmitted by the plasmodiophorid, *Polymyxa betae* Keskin. Fields remain infested with BNYVV indefinitely in *P. betae* cystosori that remain dormant up to 25 years (Abe and Tamada, 1986), therefore rotation to non-host crops or lengthening rotations is ineffective at reducing disease incidence (Rush *et al.*, 2006), and the only viable means of control has been natural resistance.

The first source of natural resistance was discovered in 1983 and is now known as *Rz*1 (Lewellen *et al.*, 1987; Scholten *et al.*, 1997). This gene, now incorporated into most major sugar beet breeding lines, confers strong resistance to BNYVV pathotypes A and B (Kruse *et al.*, 1994). However, other isolates, such pathotype P found in France and England (Koenig *et al.*, 1997; Harju *et al.*, 2002) and pathotype IV found in the Imperial Valley of California (Liu *et al.,* 2005) are not controlled by *Rz*1 resistance and are becoming an increasing threat. Additional sources of resistance have been identified, known as *Rz*2 (Scholten *et al.*, 1997; 1999), *Rz*3 (Gidner *et al.*, 2005) and *Rz*4 (Grimmer *et al.,* 2007). While these additional sources of resistance hold promise, widespread planting of new resistance sources will likely lead to further selection favoring the emergence of additional resistance-breaking BNYVV pathotypes. Furthermore, several minor genes in addition to *Rz*1 may contribute to more enhanced resistance (Gidner *et al.*, 2005), the identity of which remain largely unknown. Until the epidemiology behind the spread of resistance-breaking isolates is understood, alternative

disease control methods and additional sources of resistance will be required to control this pathogen. The first step toward more rapid, accurate selection of resistance and creation of novel disease control methods is to gain a better understanding of the underlying mechanisms of resistance and disease. This is especially critical with *Rz*-mediated resistance, since different genetic sources map to different chromosomal positions and appear to have different underlying mechanisms (Scholten *et al.,* 1997; 1999).

Proteomics is a powerful method for investigating plant response to stimuli, detecting protein differences as a result of *de novo* production (Chen *et al.*, 2005) and post-translational modification (Kiernan, 2007). Multidimensional liquid chromatography (MDLC) is an extremely sensitive, highly reproducible method of separating proteins (Wang *et al.*, 2005). Differentially expressed proteins can be rapidly identified by comparing chromatograms derived for treated and control samples. In the current study, MDLC was used to compare proteins from healthy and BNYVV-challenged resistant (*Rz*1) and susceptible (*rz*1) sugar beet. Differentially expressed proteins were identified using tandem MALDI-TOF mass spectrometry and expression of key defense- and disease-related proteins was verified by semi-quatitative reverse transcriptase polymerase chain reaction.

Materials and Methods:

Detailed information on materials and methods employed in the studies described herein are described in Larson et al. (2008).

Results and Discussion:

Rhizomania is a destructive viral pathogen of sugar beet. There are limited sources of resistance for use against BNYVV and resistance-breaking isolates are becoming an increasing threat. Furthermore, evidence suggests the alternate *Rz* sources of resistance have variable mechanisms (Scholten *et al.,* 1997; 1999) and there are minor gene contributors to resistance that remain unknown (Gidner *et al.,* 2005). A greater understanding of the mechanisms underlying resistance and disease will help to identify markers for use in more rapid, accurate molecular selection, aid in the development of novel disease control strategies and provide a framework for understanding the breakdown of certain sources of resistance in sugar beet. Our initial efforts in this process, outlined in this manuscript and in Larson et al. (2008), were to characterize sugar beet proteins affected by a non-resistance breaking isolate (A-type) of BNYVV in the most widely deployed source of resistance, *Rz*1. This response was compared to a nearly isogenic line of sugar beet lacking resistance to BNYVV.

Prior to protein analysis, the presence/absence of BNYVV in the two genotypes was verified by ELISA assay (Larson et al., 2008). Subtractive analysis of MDLC data proved to be a highly reproducible method for detecting sugar beet proteins qualitatively and quantitatively affected by BNYVV. Of the 66 total protein fractions containing differentially expressed peaks from the resistant genotype, 35 peaks eluted at concentration levels that make identification with MALDI-TOF/TOF mass spectrometry difficult, therefore only 31 were subjected to further analysis. Similarly, only 50 of the 86 differentially expressed peaks in the susceptible genotype were at levels conducive to analysis by MALDI-TOF/TOF. Sugar beet is not well represented in public databases therefore several approaches were necessary for protein identification. For all proteins, first attempts at protein assignment were completed using Mascot analysis software searching with combined peptide mass fingerprint (PMF) and MS/MS spectra. Additional identification with Mascot was achieved by running MS/MS spectra individually against the non-redundant database. Lastly, identification of the remaining proteins was attempted through homology-based searching with the *de novo* peptide sequence derived from the MS/MS spectra using PEAKS (Detailed in Larson et al., 2008). Using all three approaches, 65 proteins were identified with greater than 90% confidence. All protein identifications were furthered validated

by comparing the degree of homology of the peptide sequence across five diverse plant species (data not shown). Furthermore, proteins identified with single peptide matches were only accepted if the matched sequence was located within a highly conserved functional domain as determined using NCBI's conserved domain database.

In total, 7.4% and 11% of the entire proteome detected in this current study was affected by BNYVV for the resistant and susceptible genotype, respectively. Protein expression was temporally regulated. With the resistant genotype, 22 and 29 proteins were uniquely affected at 3 and 6 weeks, respectively and 15 were coordinately affected at both time points. Likewise, 24 and 50 proteins were uniquely affected at 3 and 6 weeks, respectively in the susceptible genotype and 12 were coordinately affected at both time points. For the resistant genotype, at 3 weeks post germination, 10 of the 22 uniquely affected proteins were induced and 12 were repressed and at 6 weeks post germination, 18 were induced and 11 repressed. For the susceptible genotype, at 3 weeks post germination 11 proteins were induced, 13 repressed and at 6 weeks post germination, 26 were induced and 24 repressed. Lastly, 11 proteins were similarly affected in both genotypes.

Only 42% of the differentially expressed proteins were able to be identified based on correlation of the acquired spectra to proteins in the public database. Although limited due to limited knowledge of the beet genome, this information provided a great deal of information regarding changes that occur in the beet proteome in response to BNYVV infection and resistance. Subtractive proteomics demonstrated the protein response to BNYVV was fairly limited, only 7.4% and 11% of the entire proteome showed reproducible differential expression following BNYVV challenge for the resistant and susceptible genotype, respectively. The protein response was also temporally regulated as illustrated in Figure 3. Not surprisingly, several of the proteins differentially affected by BNYVV in both the resistant and susceptible genotype are related to protein expression and turnover (Larson et al., 2008).

Several proteins affected by BNYVV are classically associated with plant defense, suggesting inducible resistance may contribute to viral disease suppression. These include pathogenesis-related proteins, such as chitinase, protease, glucanase, peroxidase and defensin. Interestingly, induction of these proteins was not always limited to the resistant genotype. Li *et al.* (2006) noted significant overlap between resistant and susceptible tomato following challenge with powdery mildew, half of which were more rapidly induced in the resistant line. In the current study, similar observations were made. Chitinase, defensin and protease were induced in both genotypes with increased expression occurring more rapidly in the resistant line. This suggests timing of expression is critical to defense. When examined at the transcript level, significant induction of chitinase was limited to the resistant line. Transcript expression was transient, but protein levels were sustained at increased levels through 6 weeks post germination, suggesting low protein turnover (Larson et al., 2008). Some oxidative enzymes, which are also known to contribute to plant defense appear to have similar timing-dependent expression. Polyphenol oxidase (PPO), a protein responsible for physical barrier development, and toxic compound and reactive oxygen production (Li and Steffens, 2002) is more highly and rapidly expressed in the resistant genotype when compared with expression patterns from the susceptible genotype. However, another protein peak also identified as PPO was repressed in both treatments following BNYVV challenge. Since protein modifications were not examined and molecular weights are unable to be determined using MDLC, the apparent contradictory expression noted between the three proteins might be explained as the latter PPO being modified in the challenged plants, causing a significant retention time or isoelectric point shift. The importance of PPO in resistance was confirmed by RT-PCR since the gene was induced in the resistant line and repressed in the susceptible (Larson et al., 2008). This is similar to the findings with oxalate oxidase (OXO), a protein which produces hydrogen peroxide (Hu *et al.,* 2003) which can act as a messenger for defense gene activation. Two protein peaks

identified as OXO were more highly induced in the resistant line than the susceptible. This expression pattern was further confirmed by RT-PCR (Larson et al., 2008).

Rhizomania is associated with development of a "hairy root" phenotype symptomatic of infection. The proliferation of lateral roots creates a major constriction of taproot growth, the main sucrose storage site in beet. Therefore, the symptoms of viral infection greatly reduce sugar accumulation in beet. To date, the specific physiology responsible for hairy root development remains unknown. This proteomic investigation has uncovered several potential clues regarding the cause of BNYVV-induced symptoms. Several phytohormone-related proteins induced in the susceptible line were repressed or had no noticeable change in expression in the resistant genotype. The most prevalent phytohormone correlation was with auxins although there were ethylene responsive transcription factors (Sohn *et al.,* 2006) and abscisic acid (ABA) associated proteins induced as well. In an attempt to further evaluate the role of auxin and ABA in hairy root development, RT-PCR of several contributors to the auxin signal transduction cascade (AGC2-2 and PIP) and auxin-inducible (GH3, SAUR, auxin-inducible transcription factor) genes and ABA signaling (ABA kinase) and inducible (ABA protein) proteins was performed. Results with the signal transduction components were inconclusive in both instances (data not shown). This is not surprising since most are regulated through phosphorylation cascades (Anthony *et al.,* 2006; Rossi *et al.,* 1998). The others had slight increases following BNYVV challenge, but the current methods were not sensitive enough to determine statistical differences between treatments. To further evaluate the role of these phytohormones in hairy root development, future investigations will include quantification of phytohormone levels in the beet roots as well as isolation and identification of phosphorylated proteins in the susceptible genotype.

This characterization of the sugar beet proteomic response to BNYVV has uncovered the potential contribution of systemic resistance and phytohormone activity in defense and symptom development, respectively, providing a better understanding of resistance and disease mechanisms in sugar beet. More detailed information on these studies can be found in Larson et al. (2008). Future investigations will include comparing the host protein response in *Rz*1 beet to the responses of other *Rz* resistance sources in beet, as well as the differential host response to resistance-breaking pathotypes of BNYVV. In the quest to develop more effective means of screening for resistance, the transcripts for the proteins with differential expression limited to the resistant line (chitinase, polyphenol oxidase, and oxalate oxidase) will be examined for future exploitation as biomarkers for resistance screening. Furthermore, over expression of these resistance-specific genes will be examined as a potential novel disease control mechanism.

References:

Abe, H., and Tamada, T. 1986. Association of *Beet necrotic yellow vein virus* with isolates of *Polymyxa betae* Keskin. Ann. Phytopathol. Soc. Jpn. 52: 235-247.

Anthony, R.G., Khan, S., Costa, J., Pais, M.S., and Bogre, L. 2006. The Arabidopsis protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. Journal of Biological Chemistry. 281:35736-37546.

Chen, R., Pan, S., Brentnal, T.A., and Aebersold, R. 2005. Proteomic profiling of pancreatic cancer for biomarker discovery. Molecular and Cellular Proteomics. 4: 523-533.

Gidner, S., Lennefors, B.L., Nilsson, N.O., Bensefelt, J., Johansson, E., Gyllenspetz, U., and Kraft, T. 2005. QTL mapping of BNYVV resistance from the WB41 source in sugar beet. Genome. 48: 279-285.

Grimmer, M.K., Trybush, S., Hanley, S., Francis, S.A., Karp, A., and Asher, M.J. 2007. An anchored linkage map for sugar beet based on AFLP, SNP< and RAPD markers and QTL mapping of a new source of resistance to *Beet necrotic yellow vein virus*. Theor. Appl. Genet. 114: 1151-1160.

Harju, V.A., Mumford, R.A., Blockley, A., Boonham, N., Clover, C.R.G., Weekes, R. et al. 2002. Occurrence in the United Kingdom of *Beet necrotic yellow vein virus* isolates which contain RNA 5. Pant Pathol. 51: 811.

Hu, X., Bidney, D.L., Yalpani, N., Duvick, J.P., Crasta, O., Folkerts, O., and Lu, G. 2003. Overexpression of a gene encoding hydrogen-peroxide generating oxalate oxidase evokes defense responses in sunflower. Plant Physiology. 133: 170- 181.

Kiernan, U.A. 2007. Quantitation of target proteins and post-translational modifications in affinity-based proteomics approaches. Expert Reviews in Proteomics. 4: 421-428.

Koenig, R., Haeberle, A.M., Commandeur, U. Detection and characterization of a distinct type of *Beet necrotic yellow vein virus* RNA 5 in a sugarbeet growing area in Europe. 1997. Arch. Virol. 142: 1499-1504.

Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A.G., et al. 1994. Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of Beet necrotic yellow vein virus. J. Gen . Virol. 75: 1835-1842.

Larson, R.L., Wintermantel, W.M., Hill, A., Fortis, L., and Nunez, A. 2008. Proteome changes in sugar beet in response to *Beet necrotic yellow vein virus*. Physiol. Mol. Plant Pathol. 72: 62-72.

Lewellen, R.T., Skoyen, I.O., Erichsen, A.W. 1987. Breeding sugarbeet for resistance to rhizomania: Evaluaton of host-plant reactions and selection for and inheritance of resistance. In Proc. of the IIRB 50th Winter Congress, Brussels, Belgium, 11-12 February 1987. Brussels: Institut International de Recherches Betteravieres. Pp. 139-156.

Li, C., Bai, Y., Jacobsen, E., Visser, R., Lindhout, P., and Bonnema, G. 2006. Tomato defense to the powdery mildew fungus : differences in expression of genes in susceptible, monogenic- and polygenic resistance responses are mainly in timing. Plant Molecular Biology. 62: 127-140.

Li, L., and Steffens, J.C. 2002. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta. 215: 239-247.

Liu, H.-Y., Sears, J.L., Lewellen, R.T. 2005. Occurrence of resistance-breaking Beet necrotic yellow vein virus of sugarbeet. Plant Dis. 89: 464-468.

Rossi, M., Carrari, F., Cabrera-Ponce, J.L., Vasquez-Rovere, C., Herrera-Estrella, L., Gudesblat, G., and Iusem, N.D. 1998. Analysis of an abscisic acid (ABA)-responsive gene promoter belonging to the Asr gene family from tomato in homologous and heterologous systems. Mol Gen Genetics. 258:1-8.

Rush, C.M., Liu, Y.Y., Lewellen, R.T., and Acosta-Leal, R. 2006. The continuing saga of rhizomania of sugar beets in the United States. Pant Dis. 90: 4-15.

Scholten, O.E., Klein-Lankhorst, R.M., Esselink, D.G., De Bock, T.S.M., and Lange, W. 1997. Identification and mapping of random amplified polymorphic DNA (RAPD) markers linked to resistance against *Beet necrotic yellow vein virus* (BNYVV) in *Beta* accessions. Theor. Appl. Genet. 94: 123-130.

Scholten, O.E., De Bock, T.S.M.,. Klein-Lankhorst, R.M., Lange, W. 1999. Inheritance of resistance to *Beet necrotic yellow vein virus* in *Beta vulgaris* conferred by a second gene for resistance. Theor. Appl. Genet. 99: 74—746.

Sohn, K.H., Lee, S.C., Jung, H.W., Hong, J.K., and Hwang, B.K. 2006. Expression and functional roles of the pepper pathogen-inducible transcription factor RAV1 in bacterial disease resistance, and drought and salt stress tolerance. Plant Mol. Biol. 61: 897-915.

Tamada, T., and Baba, T. 1973. *Beet necrotic yellow vein virus* from rhizomania-affected sugar beet in Japan. Ann Phytopathol. Soc. Jpn. 39: 325-332.

Wang, Y., Yang, L., Xu, H., Li, Q., Ma, Z., and Chu, C. 2005. Differential proteomic analysis of proteins in wheat spikes induced by *Fusarium graminearum*. Proteomics. 5: 4496-4503.