BIOLOGICAL AND MOLECULAR STUDIES ON POTATO-VIRUS INTERACTIONS USING *POTATO VIRUS S* AND *POTATO VIRUS Y* AS MODEL SYSTEMS

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ABSTRACT

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Potato (*Solanum tuberosum* L.) is a staple food in the world. *Potato virus Y* (PVY; *Potyvirus: Potyviridae*) is an important virus that affects yield and quality of potatoes. PVY exists as biologically distinct strains: PVY-N induces systemic veinal necrosis in tobacco, PVY-O causes hypersensitive response (HR) in potato cultivars carrying the Ny gene and PVY-NTN produces necrotic rings on the tubers of sensitive potato cultivars. The vsiRNA profiles of three distinct PVY strains, ordinary (PVY-O), tobacco-veinal necrotic (PVY-N) and tuber-necrotic (PVY-NTN) strains were determined in potato cv. Russet Burbank. The frequency and distribution of vsiRNAs varied among different strains. PVY-NTN infected plants accumulated the highest population of PVY-vsiRNAs in comparison to plants infected with PVY-O and PVY-N. In PVY-infected plants, the 21 nt class was predominant whereas in healthy potato plants 24 nt class had the maximum population. VsiRNAs were derived from every nucleotide position of the PVY genome and certain hotspots were identified which produced relatively more vsiRNAs.

Additionally, six novel miRNAs were found in PVY-infected plants. *Potato virus S* (PVS; *Carlavirus: Betaflexiviridae*) is another important potato virus distributed worldwide. The outcome of mixed infection of PVS and PVY was studied under controlled conditions in three potato cultivars, Defender, Desiree and Russet Burbank. Results showed that PVS has an antagonistic effect on PVY replication in mixed infection. The antagonistic effect was associated with less severe symptoms in dual infections as well as reduced PVY multiplication. Symptoms of PVS were not visible in Desiree and Russet Burbank plants except Defender which showed bronzing spots on the leaves. PVY symptoms included mosaic, mottling and leaf drop. It was found that the antagonistic effect of PVS on the replication of PVY is independent of host genetic background as the same pattern was found in all three potato cultivars. Potato cultivars such as Desiree carrying the Ny gene show HR to infection with the ordinary strain of PVY. In comparison to Russet Burbank, PVY levels in Desiree decreased with increasing number of days post-inoculation. The HR was found to be specific to PVY-O and was not elicited by infection by PVY-N or PVY-NTN.

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CHAPTER ONE

BIOLOGICAL AND MOLECULAR STUDIES ON POTATO-VIRUS INTERACTIONS USING *POTATO VIRUS S* AND *POTATO VIRUS Y* AS MODEL SYSTEMS

GENERAL INTRODUCTION

Potato (*Solanum tuberosum* L.) from the family Solanaceae is the fourth important food crop in the world after wheat, rice and maize. The family Solanaceae includes other important plant species like tomato and eggplant (UNFAO, The International Year of The Potato, 2008). Potato has an origin in Peru and Bolivia, where they were grown 7,000-10,000 years ago by Inca Indians. The introduction of potatoes outside Andes traces back four centuries ago when the Spanish conquerors took potato tubers with them to Europe (Spooner *et al*., 2005 and Francis 2005). There are two subspecies of potato: Andean and tuberosum. Potatoes were introduced in Europe either through sailors and through British isles around 1588 and 1593 (Reader, 2008). Large scale cultivation of potato in Europe started after 1750's. The crop was favored by farmers over other crops due to its cheapness, easiness to grow and the bulk yields. By the mid 1840's potato crop occupied one third of the arable land in Ireland (Abel, 1986). China, India, Russia, Ukraine and United States are top five potato producing countries in the world (FAOSTAT, 2013). In United States, potatoes are sometimes referred to as Irish potatoes. Potato vines grow about 60 cm tall depending on cultivar. Different varieties/cultivars produce different color flowers; white, yellow, purple and red (Simpson and Weiner, 1989; Winch, 2006). In US, seed potatoes are produced in 15 states due to their cold winters and sunny days to kill pathogens and to get disease free seed tubers (United States Potato Board, 2007). Potatoes are a good source of carbohydrates, minerals, carotenoids and natural phenols. Potatoes have resistant starch that acts

as fiber in the digestive tract and thus prevents against colon cancer (Englyst, 1992). Tuber formation in potatoes is dependent on day length and short days result in the initiation of tuber formation (Virginia, 2001).

POTATO GENOME

Potato (*Solanum tuberosum* L.) has 12 chromosomes in haploid form (840 million base pairs). The commercially cultivated potato is tetraploid (4n=48 chromosomes). Wild species of potato are diploid with (2n=24 chromosomes) and these include *S. stenomonum*, *S. phureja*, *S. goniocalyx* and *S. ajanhuiri* whereas triploid potatoes have 36 chromosomes (3n=36 chromosomes). The potato genome is highly heterozygous that makes breeding really difficult (Spooner and Hijmans 2001; Spooner and Raker, 2002, Visser *et al*., 2009). In 2001, Potato Genome Sequencing Consortium was started to sequence potato genome, a collaborative effort between fourteen countries and 28 research groups. First potato clone sequenced was doubled-monoploid (DM) DM1- 3 516 R44. The study reported, 39,039 genes in the potato genome and location of these genes on the chromosomes is now known. This information about different aspects will be helpful in potato breeding programs and for researchers working on potato diseases. The genetic diversity in cultivated potato is very high due to its polyploidy nature and the unraveling of the potato genome now paves the way for genetic studies of potato, *Solanum tuberosum* L. This will help to improve its yield, quality as well as resistance to different pathogens around the world (The Potato Genome Sequencing Consortium, 2011). Different 11 R genes have been introgressed into cultivated potato from *Solanum demissum* against *Phytophthora infestans.* Gene content and organization of potato resembles with other members of the Solanaceae including tomato, eggplant and ornamental petunia. Nineteen genes for resistance to viruses, bacteria, fungi and nematodes have been identified on potato chromosomes

and fourteen resistance genes are located in five hotspots in potato genome (Hamalainen *et al*., 1997; Cockerham, 1970; Gebhardt and Valkonen, 2001).

POTATO PRODUCTION IN UNITED STATES

The introduction of potatoes in United States is in the 17th century. In US, potato production occurs in 36 states out of 50 states. Major potato production comes from Idaho, Washington, Wisconsin, Colorado and Maine. In US, Idaho, Washington, Colorado, Maine, Wisconsin, California, Minnesota, Michigan, Nebraska, North Dakota, Oregon, are top potato producing states. In 2012, 1,149,000 acres of potato were planted in different states which gave 467,203,000 cwt with a farm value of $8.38 per cwt (USDA-NASS, 2013). In Idaho 345,000 acres were planted under potatoes in 2012 which gave an average yield of 416 cwt. Russets are very popular in Idaho with Russet Burbank being the main cultivar grown in Idaho. Over 30 different potato varieties are grown in different places in Idaho, with 100 days in Treasure Valley in Western Idaho that have the warmest mean temperatures in North-West and about 180 frost free days (USDA-NASS, 2013; Idaho Potato Commission, 2013).

In Washington State, potatoes are planted in spring and harvested in fall. In 2012, 164,000 acres of land were planted under potatoes with an average yield of 59,500 pounds per acre. In Washington State, potato production is mainly concentrated in the Columbia Basin, Yakima Valley and Skagit Valley. Potato is an important crop in Washington State, which provides $734 million to Washington State with a total value of $4.6 billion to the state. Potato industry provides jobs to 23,500 people in the state. The three seasons between plantings provide enough time for the pathogens to die out in sunshine. During growing period of the crop, farmers are advised through scientists about watering schedule because potato plants can survive without

water but drought- stressed plants produce fewer potatoes. Late blight is very destructive disease in the Washington State and farmers are advised to spray their crops with fungicides to avoid the attack of late blight fungus. Harvesting of potatoes in Washington State starts in July and extends throughout October, depending on location and variety. Varieties being grown in Washington include Russets, yellows and reds (USDA-NASS, 2013; Washington State Potato Commission, 2012).

POTATO DISEASES

The potato crop is infected by numerous bacteria, fungi, nematodes, viruses and viroids. Different fungal diseases of potato include: black dot, black pit, cercospora leaf blotch, charcoal rot, common rust, deforming rust, early blight, fusarium wilt, gray mold, late blight, leak, phoma leaf spot, pink rot, pleospora herbarum infection, powdery mildew, powdery scab, rhizoctonia canker, black scurf, verticillium wilt, wart and white mold. Among bacterial diseases, bacterial ring rot, black leg, aerial stem rot, tuber soft rot, brown rot, bacterial soft rot, common scab, dickey solani, pink eye, and zebra chip; nematode diseases: potato cyst nematode, root-lesion nematode, potato root-knot nematode, sting nematode and stubby-root nematode; among phytoplasmas: aster yellows and witches-broom. Several viruses infect potato which include: *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV), *Potato virus X* (PVX), *Potato virus S* (PVS), *Potato moptop virus* (PMTV), *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Tobacco rattle virus* (TRV), *Tobacco mosaic virus* (TMV), *Potato virus A* (PVA), *Tomato spotted wilt virus* (TSWV) Potato spindle tuber viroid (PSTVD) (Stevensen *et al*., 2001; Salazar, 1996). *Potato virus Y* (PVY, Genus *Potyvirus* and Family *Potyviridae*) is an important

virus infecting potatoes throughout the world resulting in yield and quality losses of the produce (Ward and Kushla, 1991). Genus *Potyvirus* is the largest of plant infecting viruses that includes more than 128 approved and 89 possible species (Fauquet *et al*., 2005). PVY infects a wide range of plants from 14 different genera of the Solanaceae family including potato, tobacco, tomato, eggplant and chilli (Kerlan, 2006). Different strains of PVY can be recognized on the basis of symptoms; nucleotide sequences and with serological assays (Ellis *et al*., 1997; Kerlan *et al*., 2006 and Singh *et al*., 2008). The most prevalent strain of PVY in North American potato production areas is ordinary strain (PVY-O) that induces leaf mottling, mosaic and systemic stunting in infected potato plants. Russet Burbank is the widely cultivated potato cultivar in Pacific Northwest shows systemic mosaic, mottling and plant stunting due to PVY-O infection (Figure, 3) (Gray *et al*., 2010). In *Nicotiana benthamiana* and *Nicotiana tabacum*, ordinary strain produces mosaic and leaf mottling (Figure 2). The development of symptoms is dependent on potato cultivar as well as environmental conditions. The strain PVY-O was named because it induces hypersensitive response (HR) in potato cultivars carrying the Ny gene whereas the isolates which overcame HR in potato genotypes carrying the Ny gene (Jones 1990). The avr gene products released by PVY-O trigger HR response in Desiree. In the first phase of HR, an efflux of hydroxide and potassium ions outside the cells takes place whereas in the second phase of HR, an oxidative burst in cells involved in HR occurs that leads to the death of cells (Mathews, 2007). The PVY-N strain produces systemic veinal necrosis in *Nicotiana tabacum* (Figure 7). Likewise, strain PVY-C induces HR in potato cultivars carrying the Nc gene and another group that did not fit PVY-N group on the basis of veinal necrosis in tobacco and necrotic reactions in differential potato cultivars was named as PVY-Z. PVY-Z induces hypersensitive reaction in potato cultivars carrying the Nz gene (Jones 1990, Singh *et al*., 2008).

Another isolate of PVY was named as PVY-NTN due to the development of necrotic rings on the tubers of susceptible potato cultivars and necrosis in *N. tabacum* (Beczner *et al*., 1984) (Figure 5). The NTN strain is thought to have originated as a result of recombination and mutation events. The necrotic strains (N, NTN, N:Wi) not only cause reductions in yield but they also cause quality reductions in potato tubers by inducing necrotic rings on the tubers of infected potato varieties resulting in a disease called potato tuber necrotic ringspot disease (PTNRD). A study found that European isolates of N and NTN groups are 99% identical to those present in North America (Beczner *et al*., 1984; Lorenzen *et al*., 2006).

PVY genome is positive sense, single-stranded RNA, non-enveloped, with filamentous particles of 680-900 nm in length and 11-15 nm in width. The virions are composed of 2000 copies of capsid protein (CP), which encapsidates its genome. The genome has a 5- terminal VPg (virus protein genome-linked) and a 3-terminal poly A-tail. The positive sense genome acts directly as messenger RNA whereas the VPg is a virulence determinant (Edwardson, 1947; Daughtery and Carrington, 1988; Vandervluget *et al*., 1989). The translation is initiated by recognition of the internal AUG codon by leaky scanning mechanism. The genome of PVY has one large open reading frame that is translated into one large polyprotein 350 kDa and is subsequently cleaved into ten mature functional polyproteins by three virus encoded proteases (P1, Hc-Pro and NIb). The nine cleaved proteins are P1, helper-component protease (Hc-Pro), P3, 6K1, cytoplasmic inclusion (CI) protein, nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb), genome-linked viral protein (VPg) and coat protein (CP) (Figure 1). These nine cleaved proteins perform different functions in virus life cycle (Urcuqui-Inchima *et al*., 2001; Fauquet *et al*., 2005). The helper component and coat protein is involved in aphid transmission of potyviruses by their aphid vectors. The 5' leader sequence has an internal ribosome entry site

(IRES) and cap-independent translation regulatory elements (CIREs). The IRES directs cap independent translation through a mechanism similar to that used by eukaryotes (Ravers *et al*., 1999; Torrance *et al*., 2006).

POTATO VIRUS S **(PVS)**

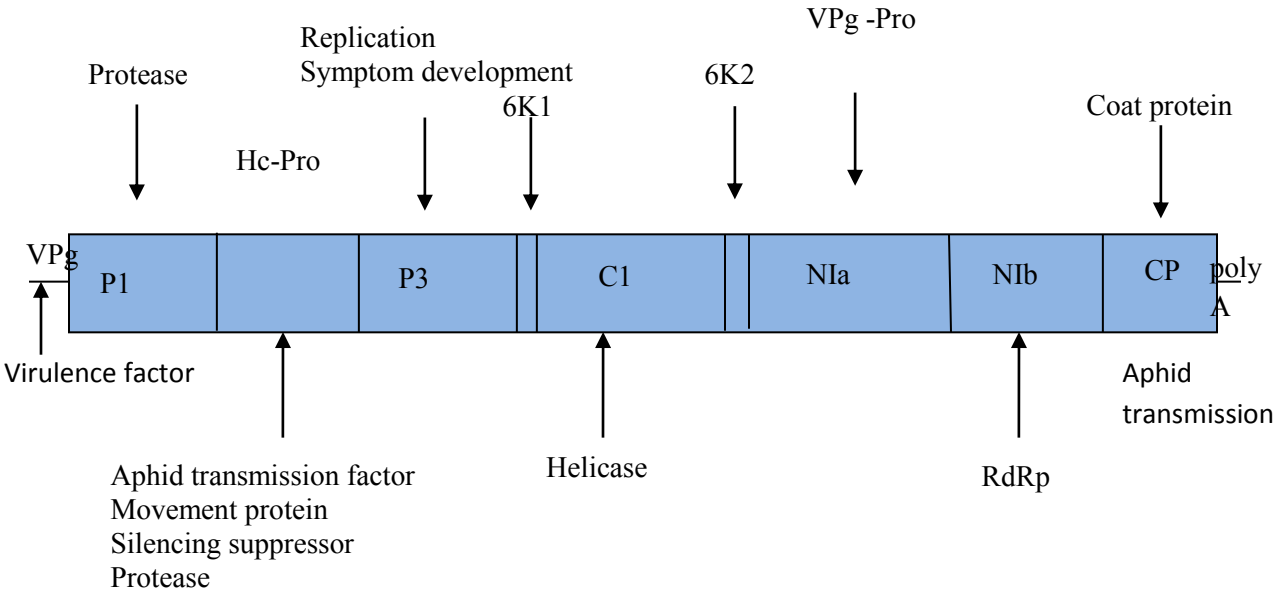
Potato virus S (PVS, Genus *Carlavirus*, Family *Betaflexiviridae*) is another important potato virus distributed worldwide (Wetter, 1971). PVS has single-stranded positive sense RNA genome of 8.5 kb in length. Transmission of PVS is through aphids in a non-persistent manner, through grafting and infected seed tubers. PVS infects pepino, potato and experimental indicator hosts from Chenopodiaceae and Solanaceae families (DeBokx 1970; Dolby and Jones, 1987). PVS is symptomless in majority of potato cultivars and many plants express mature plant resistance. Symptoms produced by PVS in infected plants include leaf rugosity, vein deepening, leaf mottling and 20% yield losses (Wetter, 1971). The virus exists as two distinct strains which differ from each other in the symptoms they produce in potato cultivars. PVS-A (Andean strain) produces more severe symptoms in potato cultivars in case of secondary infection and is present at higher concentrations in leaves. The ordinary strain does not produce visible symptoms in majority of potato cultivars with occasional symptoms of vein deepening and rugosity (Figure 6) (Rose 1983; Dolby and Jones, 1987; Jeffries 1998). PVS has flexuous particles which are 650x12 nm and the genome contains six open reading frames which encode polyproteins of 11kDa, 33kDa (coat protein) 7kDA, 12kDa, 25kDA and 41kDa respectively. The genome has 5'-cap and 3'-end poly-A tail. The viral RNA is encapsidated in an Mr. 3300 coat protein into flexous particles which are 650 nm by 12 nm (Wetter, 1971). This latter ORF encodes amino acid sequences similar to those of putative viral replicase genes and the Mr. 32515 polypeptide has been shown to be the virus coat protein (Figure 1). Nucleotide sequence data from PVS showed

that the genome organization very similar to *Potato virus X* and some other potexviruses (Mackenzie *et al*., 1989).

The second chapter deals with the comparsion of small RNA profiles from potato plants infected with different strains of PVY. The objective of the study was to analyze and characterize small RNAs from Russet Burbank plants infected with three biologically distinct and destructive PVY isolates (O, N and NTN). Necrotic isolates N and NTN produce very mild symptoms or no symptoms in infected potato plants but they produce systemic veinal necrosis in *N. tabacum* plants. Likewise, ordinary strain produces visible foliage mosaic and mottling in plants after infection (Rykbost *et al*., 1999, Nolte *et al*., 2002; Boonham *et al*., 2002). These biological differences of sympom induction of necrotic isolates in potato versus tobacco provoked us to have a deep understanding of the underlying mechisms in virus-host interactions. These small RNAs can be divided into short interfering RNAs (siRNAs) and microRNAs. Strainal profiles of small RNAs in plants infected with different strains will indicate that the symptoms development is associated with increased disease and vice versa. The findings are very useful in understanding the interaction of viruses and viral strains in the same host.

The third chapter deals with the study of interaction between PVS and PVY in three potato cultivars. Mixed infections of potato viruses are very common and the viruses interact with each other resulting in different disease phenotypes. The interactions are sometimes dependent on the host and cultivar but sometimes the interaction is independent of the host genetic background. Different potato cultivars are grown in the Pacific Northwest and in my study; I used three popular potato genotypes: Russet Burbank, Defender and Desiree.

Fourth chapter is about the symptoms produced by ordinary strain of PVY (PVY-O) in potato cultivar Desiree. Desiree is the main crop potato cultivar and is grown in the Pacific Northwest. Due to the presence of dominant Ny gene Desiree shows hypersensitive response (HR) to infection with ordinary strain of PVY. The HR is associated with increased with the development of necrotic spots in infected plants that limit the spread of pathogen further in the plant.



(Winterhalter, 2005) (http://www.uq.edu.au/vdu/VDUPotyvirus.htm)

Figure 1: Genome organization of *Potato virus Y.*

Figure 2: *Nicotiana benthamiana* plant showing systemic mosaic 2 weeks post- inoculation with ordinary strain of *Potato virus Y* (PVY-O)

Figure 3: Symptoms of ordinary strain *of Potato virus Y* (PVY) on Russet Burbank plants; (A) systemic mosaic as a result of infection with ordinary strain (PVY-O), (B) systemic stunting and leaf crinkling due to PVY-O infection.

Figure 4: Chlorotic lesions on leaves of *Chenopodium quinoa* 21 days-post inoculation with *Potato virus S* (A) PVS symptoms on leaves of inoculated plants 21 days post-inoculation (B) leaves of healthy *Chenopodium quinoa*.

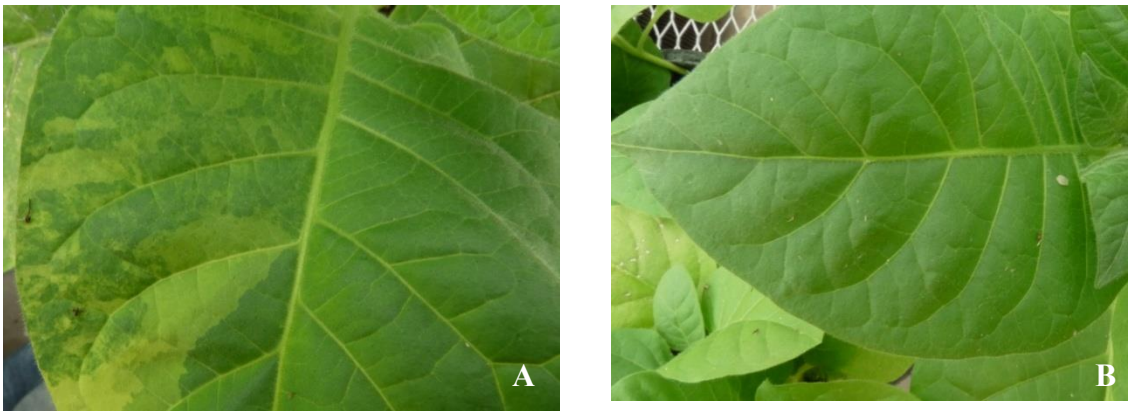


Figure 5: *Nicotiana tabacum* plant showing symptoms associated with tuber necrotic strain of *Potato virus Y*. (A) leaf of *N. tabacum* showing PVY-NTN symptoms and (B) healthy leaf of *N. tabacum*

Figure 6: Potato plants showing symptoms caused by *Potato virus S*. (A) Defender leaf showing bronzing spots and (B) healthy leaf.

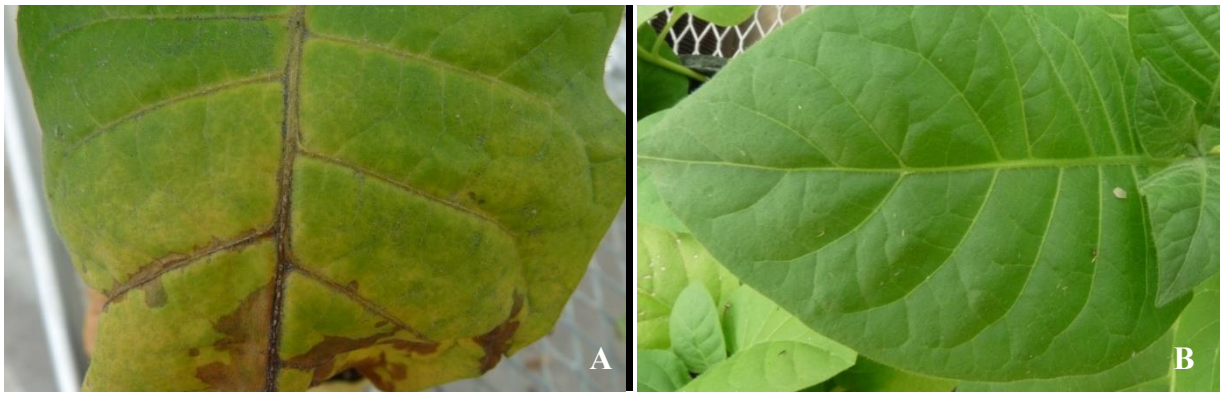


Figure 7: *Nicotiana tabacum* plants showing symptoms caused by *Potato virus Y*-tobacco veinal necrotic strain (PVY-N) (A) leaf with veinal necrosis three weeks post-inoculation, (B) healthy leaf.

REFERENCES:

Abel WA. (1986). Agricultural Fluctuations in Europe: From the Thirteenth to the Twentieth Centuries. Taylor and Francis. ISBN 0-312-01465-1.

Beczner L., Horvath J., Romhanyi I., and Foorster H. (1984). Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Research 27: 339–3.

Ellis P., Stace-Smith R., and de Villiers G. (1997). Identification and geographic distribution of serotypes of *Potato virus Y*. Plant Disease 81: 481-484.

Englyst HN, Kingman SM, Cummings JH (1992). Classification and measurement of nutritionally important starch fractions. European Journal of Clinical Nutrition. 46: S33–S50.

Fauquet CM., Mayo MA., Maniloff J., Dessel berger U, and Ball LA. (2005). Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. Elsevier Academic.

Fletcher JD. (1996). Potato virus SA…….characteristics of an isolate from New Zealand. New Zealand Journal of Crop and Horticultural Science. 24: 335-339.

Foster GD. (1992). Molecular variation between ordinary and Andean strains of Potato virus S. Research in Virology 142: 413-416.

Foster GD and Mills P. (1992). The 3 nucleotide sequence of an ordinary strain of Potato virus S. Virus Genes 6: 213-220.

Galvino-Costa SBF, Figueira AR., Rabelo-Filho FAC., Moraes FHR., Nikolaeva OV and Karasev AV. (2012). Molecular typing of *Potato virus Y* isolates from Brazil reveals a diverse set of recombinant strains. Plant Disease 96: 1451-1458.

Gebhardt C and Valkonen JPT. (2001). Organization of genes controlling disease resistance in the potato genome. Annual Review of Phytopathology 39: 79-102.

Hawkes JG. (1992). History of the Potato. In 'The potato cropped. P. Harris. Champan and Hall, London.

Hijmans R. (2001). Global distribution of the potato crop. American Journal of Potato Research. 78: 403-12.

Idaho Potato Commission. The Official Home of the Idaho Potato. (2013). (http://www.idahopotato.com). Date retrieved. 11/14/2013.

International Year of The Potato. 2008. The Potato. United Nations Food and Agriculture Organization. 2009. (ftp://ftp.fao.org/docrep/fao/011/i0500e/i0500e02.pdf) Date retrieved. 11/14/2013.

Jeffries CJ. 1998. Potato. FAO/IPGRI. Technical guidelines for the safe management of germplasm No. 19: 62-63.

John Reader J. (2008). Propitious Esculent. The Potato in world history.

Jones RAC. (1987). Problems associated with potyviruses in potato certification - field inspection and serological testing. EPPO Bulletin 17: 61-67.

Jones RAC. (1990). Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. Annals of Applied Biology 117: 93–105.

Karasev AV., Meacham T., Hu X., Whitworth JL., Gray SM., Olsen N and Nolte P. (2008). Identification of *Potato virus Y* Strains Associated with Tuber Damage during a Recent Virus Outbreak in Potato in Idaho. Plant Disease.92: 1371.

Kerlan C. (2006). *Potato Virus Y*. CMI/AAB Descriptions of Plant Viruses 414.

Lorenzen JH., Meacham T., Berger PH., Shiel PJ., Crosslin JM., Hamm PB and Kopp H . (2006). Whole genome characterization of *Potato virus Y* isolates collected in the western USA and their comparison to isolates from Europe and Canada. Archives of Virology. 151: 1055-1074.

Mackenzie DJ, Tremaine JH and Stace-Smith R. 1989. Organization and interviral homologies of the 3-terminal portin of *Potato virus S* RNA. Journal of General Virology 70: 1053-1063.

Matthews, Ben. 2007. The Hypersensitive Response. *Agricultural Research Service: Plant Science Institute*. The United States Department of Agriculture. Retrieved 2007-01-12.

National Potato Council, (2013). Potato Statistical Yearbook. (http://nationalpotatocouncil.org/files/4613/6940/5509/2013_NPC yearbook_Web)

Potato statistical yearbook. (2013). Department of Agriculture (USDA)/National Agricultural Statistics Service (NASS).

Raker CM and Spooner DM. (2002). Chilean tetraploid cultivated potato, *Solanum tuberosum*, is distinct from the Andean populations; microsatellite data. Crop Science. 42: 1451-1458.

Rupasov VV., Morozov SYu., Kanyuka KV., Zhurina VYu., LuKasheva LI. & Zavriev SK. (1990). Nucleotide sequence and genome structure of the T-terminal region of *Potato virus M* genomic RNA. Molekulyarnaya Biologiya (USSR) 24: 448-459.

Singh RP., Valkonen JP, Gray SM, Boonham N, Jones RA, Kerlan C, and J. Schubert. 2008. Discussion paper: The naming of *Potato virus Y* strains infecting potato. Archives of Virology 153: 1–13.

Spooner DM., Mclean K., Ramsay G., Waugh R. and Bryan GJ. (2005). A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. PNAS. 102: 14694-99.

Stevenson WR, Loria France GD, Weingarten DP. Eds. (2001).Compendium of potato diseases, 2nd edition. APS press, St. Paul.

Tony W. (2006). Growing Food: A Guide to Food Production. Springer Science and Business Media. ISBN 1-4020-6624-4.

Torrance L., Andreev IA., Gabrenaite-Verhovskaya R., Cowan G., Makinen K., and ME Taliansky. (2006). An unusual structure at one end of potato potyvirus particles. Journal of Molecular Biology 357: 1–8.

United States Potato Board. (2007). (http://www.potatoesusa.com/products.php?sec). Date retrieved 11/14/2013.

Virginia A, Jordi B, Jaime M, Elena Monte, Rodriguez-Falcon M, Esther R, and Salome P, (2001). Regulation of potato tuberization by day length and gibberellins. International Journal of Developmental Biology (45): S37–S38. Retrieved 8 January 20.

Visser, RGF, Bachem, CWB, Boer, JM, Bryan, GJ, Chakrabati, SK, Feingold, S, Gromadka, R, Ham, RCHJ, Huang, S, Jacobs, JME, Kuznetsov, B, Melo, PE, Milbourne, D, Orjeda, G, Sagredo, B, Tang, X. (2009). Sequencing the potato genome: outline and first results to come from the elucidation of the sequence of the world's third most important food crop". American Journal of Potato Research 86 (6): 417–429.

Washington state Potato commission, (2012). (http://www.potatoes.com/our-industry). Date retrieved.11/10/2013.

Wetter C. (1991). *Potato virus S*.CMI/AAB. Descriptions of plant viruses, No 60, 3pp.

Ward CW and Shukla DD (1991). Taxonomy of Potyviruses. Current problems and possible solutions. Intervirology 32: 269-296.

CHAPTER TWO

COMPARATIVE ANALYSIS OF VIRUS-SPECIFIC SMALL RNA PROFILES OF THREE BIOLOGICALLY DISTINCT AND DESTRUCTIVE STRAINS OF *POTATO VIRUS Y* (PVY) FROM PVY-INFECTED POTATO (*SOLANUM TUBERSOUM*) CV. RUSSET BURBANK

ABSTRACT

Potato virus Y is an important pathogen of cultivated potatoes throughout the world. In this study, we compared populations of virus-derived small RNAs recovered from potato plants cv. Russet Burbank separately infected with three biologically distinct strains of *Potato virus Y* (PVY) - ordinary strain (PVY-O), tobacco veinal-necrotic strain (PVY-N) and necrosis tuber-necrotic strain (PVY-NTN). Results showed the presence of PVY-specific small RNAs ranging in size from 17-26 nucleotides (nt) in PVY-infected plants. Considerable differences were observed in small RNA populations belonging to different size classes in cv. Russet Burbank infected with three different strains of PVY. There was a significant bias in the generation of small RNA molecules from the plus strand of the virus genome compared to the minus strand. In PVY-infected plants, the 21 nt class was predominant followed by 22 and 24 nt classes whereas in healthy potato plants the 24 nt class was predominant. The highest number of siRNAs were found in PVY-NTN infected plants followed by those in PVY-N and PVY-O infected plants. Likewise, there were considerable differences in the populations of endogenous small RNAs in plants infected with different PVY strains. In addition to the previously reported conserved microRNAs, we identified 258 non-conserved microRNAs and several candidate miRNAs from 13 different miRNA families. We also identified 6 novel microRNAs from PVY-infected potato plants. The microRNA profiles varied among plants infected with different PVY strains.

MicroRNAs belonging to different 13 families were expressed at different levels among the three PVY strains. These findings indicate that three strains of PVY interact differently in the same host genetic background and provide information to better understand host-pathogen interactions in a staple food crop.

INTRODUCTION

Fredrick Sanger developed a method of DNA sequencing in 1977, also known as dideoxy chain termination method. DNA sequencing refers to determining the order of nucleotide bases: adenine, guanine, cytosine and thymine in a DNA molecule. In Sanger sequencing, chain elongation is terminated when a wrong terminating base is added during DNA replication that depicts the last nucleotide in the DNA chain, giving sequencing information of the whole DNA molecule (Sanger *et al*., 1977). The modern methods of sequencing aim to give higher accuracies by sequencing a genome several times and this demand has led to the development Deep Sequencing and Ultra deep sequencing technologies. Information about cell transcriptome was revealed through gene expression microarrays in 1970's but they had a disadvantage of the required prior sequence information and a probe to determine the DNA sequences present in a sample. Microarray probes are designed on the basis of prior genome inference sequence, and light intensity is used to measure gene expression, microarrays will miss exon junctions for novel expressed regions and RNA editing events cannot easily detect allele specific differences in gene expression (Maskos and Southern, 1992). Modern microarrays have multiple probes which can determine multiple genes present in the DNA or RNA sample (Heller *et al*., 1997). Inspite of providing large amounts of data instantly from diseased samples and tissues, microarrays have certain limitations (Abdulla-Sayani *et al*., 2006).

Development of Massively Parallel High Throughput Sequencing has revolutionized the field of sequencing. We can sequence multiple fly genomes in a matter of days (Pettersson *et al*., 2009). The technology allows a DNA fragment to be sequenced in short time…through deep sequencing that gives greatly increased sensitivity and accuracy. Deep sequencing is not dependent on any prior sequence information as well as it provides information about all RNA species present in a sample and allowing for discovery of novel microRNAs or other types of small RNAs (Friedlander *et al*., 2006). Deep sequencing provides an excellent tool for those species where limited sequence information is available. Additionally, new sequence information provided by this technology can be used to design microarray probe content for large scale expression studies (Sultan *et al*., 2008).

SANGER SEQUENCING

In Sanger sequencing, the single-stranded DNA to be sequenced is primed for replication with a short complementary strand at one end. The preparation is divided into four batches, and each batch is treated with a different replication-halting nucleotide, together with the four usual nucleotides. Chain terminating nucleotides lack 3-OH group without which phosphodiester bonds cannot be formed between two nucleotides. The replication reaction continues until a reaction terminating nucleotide is incorporated into the growing strand. In this way, "C" reaction produces new strands that terminate at G positions in the strand being sequenced. The reaction products are then separated though gel electrophoresis from which the sequence of the original single strand can be inferred (Sanger *et al*., 1977).

PYROSEQUENCING

The technique was developed by Mustafa Ronaghi and Pal Nyren in Stockholm in 1996 and it is based on sequencing by synthesis. Pyrosequencing is based on the emission of light from firefly enzyme luciferase that emits light upon the incorporation of a nucleotide by DNA polymerase. The amount of light emitted is dependent upon the number of nucleotides added in the chain. Single-stranded DNA template is used in the reaction and the complementary strand is synthesized. The DNA template is incubated with Polymerase, dNTPS, ATP sulfurylase, luciferase, apyrase, adenosine 5'-phosphosulfate and luciferin (Ronaghi *et al*., 1996). The technique offers greater accuracy and is widely applicable. There is no need for primers, labelled nucleotides as well as gel electrophoresis. Biologists prefer this technique due to its high reading efficiency and it is being used for high throughput analyses (Ronaghi, 2001).

IllUMINA/SOLEXA SEQUENCING

The technology is similar to Sanger sequencing but it uses modified dNTPs (deoxy nucleotide triphosphates) with a terminator to block polymerization and permit elongation of the chain. The sequencing is done on a solid platform with large number of template molecules at the same time. It is also called terminator technology or reversible chemistry. The terminator contains a fluorescent label, which is detected by camera. Only a single fluorescent color is used, so each of the four bases must be added in a separate cycle of DNA synthesis and imaging. With this technology, DNA reads of uniform length are created in each cycle. Solexa, now has joined Illumina and in 2005, Solexa acquired a technology called clusters in which DNA is clonally amplified on a surface. In this method, DNA molecules and primers are attached on a solid surface and amplified with a polymerase, so that clonal colonies called “clusters” are formed. To

determine the sequence, four types of reversible terminator bases are added and non-incorporated nucleotides are washed away. The dye along with the 3-terminal blocker is chemically removed from the DNA allowing the next cycle. Unlike pyrosequencing, the DNA chains are extended one nucleotide at a time and image acquisition can be performed at a delayed moment, allowing for large arrays of DNA colonies to be captured from a camera. This technology has become most successful worldwide during the last few years

(Illum. Incor. 2013) (http://www.illumina.com/technology/sequencing_technology.ilmn)

APPLICATIONS OF DEEP SEQUENCING IN PLANT DISEASE DIAGNOSIS AND UNDERSTANDING

In response to pathogen elicitors, plants activate secondary defense responses and gene silencing is one of these secondary defense mechanisms in which the invading pathogen is unable to transcribe its genome. As a result of this RNA silencing, small RNA molecules accumulate in infected plant cells (Ding and Voinnet, 2007). These small RNAs are characterized by their double-strandedness, two nucleotide overhangs at 5 and 3 prime ends and with a length of 20-25 nucleotides. These virus-derived short RNA molecules are also are abbreviated as vsiRNAs. The accumulation of vsiRNAs in virus-infected plants indicates that the invading pathogen was counteracted by host defense machinery into small RNAs. These small RNA molecules are derived from long messenger RNA molecules as a result of its degradation by host RNA silencing machinery (Nykanen *et al*., 2001; Burgyan and Havelda, 2011; Llave, 2010). Characterization of small-interfering RNAs (siRNAs) derived from rice leaves infected with *Rice stripe virus* (RSV) through high throughput Illumina sequencing showed equal populations of sense and antisense derived siRNAs, majority of which were 20-22 nt in length

with majority produced from the 3-terminal end of the RSV genome. Out of four genomic RNA components more siRNAs were derived from the RNA4 (Yan *et al*., 2010). Similar to viruses, infection with viroids results in the generation of small RNAs. A study performed at the Institute of Molecular and Cell Biology in Singapore investigated interaction of viroids in grapevines infected with *Hop stunt viroid* (HSV) and *Grapevine yellow speckle viroid 1* (GSVD) through high throughput sequencing. Majority of HSVD and GSVD derived sRNAs were originated from few hotspots of the genome of both RNAs with majority originating from the negative sense of the genomes. The majority of viroid derived small RNAs were from the 21, 22 and 24 nucleotide classes (Navarro *et al*., 2009).

Deep sequencing technology has made it easier to identify novel viruses from infected plants. High throughput sequencing of diseased symptomless plants resulted in the discovery of many novel DNA and RNA viruses. Through assembly of small RNA contigs viruses occurring at extremely low titers were detected (Kreuze *et al*., 2009). Similarly, another study reported five novel viruses from *Drosophila* cells and adult mosquitoes through sequencing of small RNA libraries. The identified viruses showed very little similarity to already reported viruses. Virus-derived Piwi-interacting RNAs (piRNAs) were also discovered in *Drosophila melanogaster* that have not been previously reported from other insect species (Wu *et al*., 2009).

Deep sequencing technology is being used for studying microRNA (miRNA) profiles of different plant species. Analysis of small RNA transcriptome of bread wheat (*Triticum aestivum*) and *Brachypodium distachyon* through deep sequencing revealed 70 conserved miRNAs from 25 miRNA families as well as 23 novel miRNAs. From *Brachypodium*, 12 putative miRNAs were predicted and further, 94 conserved miRNAs were identified from 28 families in bread wheat. Differential expression of miRNAs during different growth stages showed that miRNAs have

role in organ development and differentiation (Wei *et al*., 2009). In another study, small RNA profiles of viruses infecting grape vineyards in Africa were studied using deep sequencing technology. Results showed that four new viruses were present in infected grapevine samples. The viruses identified included *Grapevine leafroll-associated virus* 3 (GLRaV-3), *Grapevine rupestris stem pitting-associated virus, Grapevine virus A* and *Grapevine virus E*. GLRaV-3 was present at high rates in infected plants (Coetzee *et al*., 2010).

Small RNA profiles of cotton plants infected with *Cotton leafroll dwarf virus* were analyzed through deep sequencing. Viral small RNAs (vsRNAs) ranged size range between 21-24 nt that were originated from the whole virus genome and there was high population of vsRNAs from the 3-end of the genome. There was equal population of sense and antisense vsRNAs and the 22 nt class was the predominant class that indicated that DCL1 was upregulated in virus infected plants (Silva *et al*., 2011). In a study, virus populations from raw sewage were analyzed through deep sequencing. The data revealed 234 known viruses, out of which 17 were human infecting viruses. Viruses infecting plants, insects, and algal viruses as well as bacteriophages were also detected belonging to 26 families. The identified viruses included single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), positive-sense single stranded RNA and double-stranded RNA viruses. The viruses identified represented 51 families and it indicates that sewage water harbors the maximum population of diverse viral populations. However, the vast majority of sequence reads bore little or no sequence relation to known viruses and thus could not be placed into specific taxa (Cantalupo *et al*., 2011). Similarly, viruses infecting sweet potato in Honduras and Guatemala were studied through deep sequencing. The deep sequencing data revealed the presence of *Sweet potato feathery mottle virus* strain RC, *Sweet potato chlorotic stunt virus* strain WA, *Sweet potato leaf curl Georgia virus* and *Sweet*

potato pakakuy virus strain B. Small RNA contigs were mapped to known viruses in NCBI, which resulted in the detection of six viruses. The results suggested that Small RNA deep sequencing is a useful tool for the discovery and identification of novel viruses (Kashif *et al*., 2012).

Martinez *et al*., (2012) studied the virus populations in infected plants at frequencies below than 2 x 10^{-5} through high throughput sequencing. The evolutionary passage in host plants before and after the viral population was successfully able to infect the plants was studied. Further, they studied the amiR target sequence and they found that the every position in the amiR sequence represented variation. The study concluded that resistance in susceptible plants is a result of equilibrium between mutation and genetic drift. Wang *et al*., (2010) found that RNA interference (RNAi) mediated viral immunity requires RNA-dependent RNA polymerases in *Arabidopsis thaliana*. An Arabidopsis mutant of *Cucumber mosaic virus* (CMV-Δ2b) was studied for RNA interference and it was found that virus-derived small interfering RNAs (vsiRNAs) targeting CMV-2b require suppressor of gene silencing-3 and dicer-like-4 (DCL4) in addition to RDR6 and RDR1. RDR1 and RDR6 target specifically tripartite genome of positive sense CMV. Argonaut proteins, AGO1 and AGO2 played an essential role in defense against CMV-Δ2b in all RDR mutants. In another study, virus infected tomato samples from Mexico and United States were analyzed through sequencing in order to identify different viruses and viroids. The analysis of small-interfering RNAs (siRNAs) led to the identification of two isolates (EU and US1) of *Pepino mosaic virus* (PepMV) as well as novel virus with 70% homology to potyvirus was discovered. Further, they could assemble six genomes of PepMV from three US samples. The results showed that small RNA technology can be used for studying viruses and viroids in plants (Ling *et al*., 2011).

Zheng *et al*. (2011) compared small RNA profiles of rice plants infected with two different viruses: *Rice dwarf virus* (RDV, dsRNA virus) and *Rice stripe virus* (RSV) in comparison to healthy rice plants. In virus infected plants, the levels of microRNAs (miRNAs) were enhanced but their corresponding miRNAs were found at the same levels. In addition, several conserved miRNAs were expressed in virus infected plants. Rice plants infected with viruses did not show any differences in accumulation of small RNA profiles. Certain dicer enzymes and agronuate protein were differently expressed in rice plants infected with RDV and RSV. In another study, Xu *et al*., (2012) examined small RNA profiles of rice stripe virus infected three plant species: *Oryza sativa*, *Nicotiana benthamiana* and *Laodelphgax striatellus* by using high throughput sequencing. They observed a significant bias in the population of sense versus antisense strands of *Rice stripe virus* infected *Oryza sativa* plants whereas in other two plants species, there was equal distribution of sense versus antisense small RNA*s.* The number and size distributions of vsiRNAs in the three hosts were very significant. In *Oryza sativa* and *N. benthamiana,* more vsiRNAs mapped to the discrete regions in the RSV genome and more of the vsiRNAs from these two hosts were generated from RSV RNAs 3 and 4. In contrast, the vsiRNAs identified in *L. striatellus* distributed uniformly along the whole genome of RSV. In *L. striatellus*, they found greater accumulation of sense versus antisense viral small RNAs.

Molina *et al*. (2012) identified several fungi, bacteria and viruses in soybean samples through the analysis of small RNAs through deep sequencing. The samples were collected from soybean field plantations as well as soybeans grown in greenhouses under a controlled environment. There were differences in pathogens present in field grown and greenhouse grown soybeans. Thus deep sequencing is useful to identify unknown pathogens in plants growing in different environments. Another study was conducted in Finland by Bi *et al*., (2012) and they

studied small RNA profiles to detect viruses in woolly burdocks displaying mosaic and yellowing, typical of virus symptoms. A positive sense virus was identified through small RNA contigs similar to the genus Potexvirus and other negative sense viruses related to family Emaravirus. Nucleocapsid protein amino acid sequences of the emara-like virus showed only 78% or less similarity to emaraviruses. *Woolly burdock yellow vein virus* was identified in samples showing mosaic symptoms.

Xie *et al*., (2009) analyzed small RNA profiles of *Tobacco mosaic virus* and *Arabidopsis thalilana* through high throughput sequencing. They used wild type as well RdRp mutants if Arabidopsis to study small RNA profiles. They observed 100,000 TMVCg specific small RNA reads of 21 and 22 nucleotides in length from the samples. Certain hotspots were identified in the TMV genome which produced high number of small RNAs. RDR1 and RDR6 showed reduced activity to form small RNAs and it indicated that these polymerases have role in gene silencing. Plant-encoded RNA dependent RNA polymerases (RDR) have role in viral small RNAs (vsRNA) biogenesis. RNA dependent RNA polymerase 1 (RDR1) and RNA dependent RNA polymerase 6 (RDR6) contributed to the accumulation of vsRNAs in virus-infected cells. RDR1 was responsible for the majority of vsRNAs in plants infected with three different viruses but when RDR1 was mutated, RDR6 acted as a surrogate. RDR1 generated vsRNAs from the 5′ ends of genome whereas RDR6 generated vsRNAs mapped to 3 ends of the genome. When both RDR1 and RDR6 were absent, vsRNAs levels were diminished resulting in increased viral accumulation (Qu, 2010).

High throughput sequencing analysis in tomato fruits and leaves resulted in the identification of microRNAs (miRNAs) that showed tissue specific expression indicating that they have role in fruit development. A novel miRNA targeting CTR family involved was

identified. The results suggested that target prediction of plant small RNAs should be validated to identify their targets accurately and miRNAs may be regulating fruit development and ripening in plants (Moxon *et al*., 2008). The small RNA analysis through next generation high throughput solexa sequencing revealed complex populations in peanuts. Analysis showed that peanuts have a complex small RNAs, 24 nt class was the predominant class with majority of small RNAs. The bioinformatics analysis showed 14 novel and 70 conserved microRNAs in peanuts. All 14 novel peanut microRNAs were found to be species specific because no homologs have been found in other plant species (Zhao *et al*., 2010). Huang *et al*., (2009) studied small RNA libraries prepared from young and adult pairing *Schistosoma japonicum* by using deep sequencing technology. Through bioinformatic analysis, they identified 176 new microRNAs in *S. japonicum*. They categorized them as new microRNAs as they were not reported before in other species. They observed differential expression of microRNAs between both, young and adult stages of *S. japonicum*. The data showed that microRNAs play important role in growth and development.

VIRUS DERIVED SHORT INTERFERING RNA(s) IN VIRUS INFECTED PLANTS

Compatible virus-plant interactions usually result in the accumulation of virus derived small RNA in infected plant cells as a result of host induced defense mechanisms (Dunoyer and Voinnet, 2005). The generation of small RNAs indicates that the invading virus was challenged by host induced RNA-silencing machinery as a result of which long double-stranded viral messenger RNA (mRNA) molecules were chopped down into small double-stranded RNA molecules of 20-25 nts in length and the virus genome expression was blocked. Various host proteins and enzymes which include Dicer-like proteins and Argonaut proteins are involved in the generation of these small RNA molecules. These small RNA molecules are termed as virus

derived small RNAs or short interfering RNAs. Small interfering (si)RNAs have unique features which distinguish them from other small RNA classes. They are characterized by their double-strandedness, phosphorylated 5' ends and hydroxylated 3' ends with two nucleotide overhangs protruding at both 5' and 3' ends. During the process of gene silencing, the antisense strand of these viral small RNA duplexes (siRNA molecules) is recruited into the RNA-induced silencing complex (RISC). After getting incorporated into the RISC complex, these siRNAs selectively target and destroy complementary messenger RNA sequences, thus resulting in the silencing of homologous viral RNAs (Brodersen and Voinnet, 2006). During virus-host interactions, the production of siRNAs not only requires Dicers and Argonuate proteins but it is also dependent on virus encoded RNA dependent RNA polymerases. The RDRs derived primary siRNAs then trigger the generation of secondary siRNAs through RNA silencing or RNA interference (Wang *et al*., 2010).

Since the first discovery of microRNAs (miRNAs) in *Caenerhabditis elegans,* they have been found in unicellular prokaryotes to multicellular eukaryotes. Plant miRNAs have perfect or near perfect base pairing with their messenger RNA (mRNA) targets and they cause gene silencing via target degradation through binding in both non-coding and untranslated regions (He *et al*., 2004). MiRNAs are involved in host defense when the plants are invaded by pathogens as well as when the plants are under abiotic stress. Thus miRNAs are important in plant growth, development and defenses against pathogens. The alteration of expression pattern under stress conditions in plants provides the evidence that they have significant roles in plant growth and development (Sunkar *et al*., 2012). There are reports of miRNAs encoded by animal infecting viruses but there is no report for miRNAs encoded by plant viruses. MiRNAs encoded by hosts and viruses have several unique features and they play roles in silencing both host-encoded

transcripts as well as virus-encoded transcripts (Grundhoff and Sullivan, 2011). The evidence of direct antiviral activity of plant miRNAs is lacking, however, artificial miRNAs have been used to confer virus resistance in plants (Simon-Mateo and Garcia, 2011). During pathogen-host interactions, changes in the regulation of miRNAs have been observed and symptom alteration affecting patterns of expression of miRNAs as a result of infection with different viruses has also been observed during virus-host interactions. Alteration in the expression of viral proteins also effects microRNAs accumulation in different virus-host interactions. Studies have found that severe strains of viruses result in enhanced levels of miRNA accumulation that play roles in leaf development, morphogenesis and small RNA processing (Bazzini *et al*., 2007; Cillo *et al*., 2009; He *et al*., 2008).

POTATO VIRUS Y

Potato virus Y (PVY) is a devastating pathogen infecting potatoes worldwide causing significant yield and quality losses of the produce (Ward and Shukla, 1991). The family *Potyviridae is* currently one of the largest genera of plant infecting viruses which has 128 approved and 89 tentative species (Fauquet *et al*., 2005). PVY is a complex of different strains which differ from each other in the symptoms they produce in infected plants, host reaction and their nucleotide sequences. PVY strains include ordinary strain (PVY-O), stipple streak strain (PVY-C) and necrotic strains; tobacco veinal necrotic strain (PVY-N), necrosis tuber-necrotic strain (PVY- NTN), necrotic wigla (PVY- N:Wi) and recombinant between N and O (PVY-N:O) (Singh *et al*., 2003; Chachulska *et al*., 1997 and Kerlan *et al*., 2001). Ordinary strain of PVY is the most prevalent strain in Europe and United States and it produces visible foliar symptoms including mosaic, mottling, leaf drop and premature leaf senescence in majority of potato cultivar (Gray *et al*., 2010). The necrotic strains do not produce visible foliage symptoms in

potato plants and this result in PVY disease escapes. The necrotic strain PVY-N induces systemic veinal necrosis in tobacco and some potato cultivars. The PVY-NTN isolates have a characteristic of producing PTNRD in susceptible potato varieties but PVY-NTN does not always produce PTNRD in even if the virus infection in is field very high (Beczner *et al*., 1984). Isolates from PVY-N strain group induce systemic veinal necrosis in tobacco whereas potato cultivars carrying Nc or Ny genes do not show systemic veinal necrosis due to PVY-N infection. (Karasev *et al*., 2008 and Singh *et al*., 2008). PVY genome is single-stranded, positive sense, 9.7 kb in length and is translated into a single large polyprotein that is subsequently cleaved into 10 mature functional proteins by the action of three virus encoded proteases (Hc-Pro, P1 and Nib). These proteins are multifunctional and they perform different functions in the virus life cycle. PVY genome has an untranslated region at the 5' end and a poly A-tail at the 3' end of the genome. The virions are 680-900 nm in length and 11-15 nm in width (Edwardson, 1947) and genome of PVY has one major species of 29-kDa capsid protein, whereas Hc-Pro is also present in virus particles along with CP and VPg that is covalently attached to PVY RNA genome at the 5' end (Torrance *et al*., 2006).

To our knowledge, no information is available about the composition of virus specific small RNAs and miRNAs in potato infected with PVY. In this study, we hypothesized that different strains of *Potato virus Y* interact differently in potato plants of the same cultivar during infection process, thereby resulting in the differential expression of virus derived small RNAs as well as plant encoded miRNAs. For testing the hypothesis, the small RNA populations were obtained from PVY-infected potato plants by using three most prevalent and economically important strains of PVY through high throughput sequencing. Potato cultivar Russet Burbank was used in the study due to its wide adoption and cultivation in the Pacific Northwestern USA

and Canada. It is a multipurpose potato cultivar and is suitable for fresh market and is excellent for baking and French fries (The Potato Association of America, 2009).

MATERIALS AND METHODS

PLANT MATERIALS AND VIRUS INOCULATIONS

Three strains of PVY- ordinary strain (PVY-O), tobacco veinal- necrotic strain (PVY-N) and necrosis tuber- necrotic strain (PVY-NTN) were provided by A. Karasev, University of Idaho and were maintained in *Nicotiana tabacum* in a greenhouse (Lorenzen *et al*., 2005; Karasev *et al*., 2011; Hu *et al*., 2009). The isolates were characterized on the basis of their symptoms in indicator hosts. Healthy plants of potato cultivar Russet Burbank were grown through tissue culture from virus free potato plants and were transplanted in soil in LC1 potting mixture in insect-free greenhouse. Six week-old plants were inoculated with virus inocula by grinding the infected leaves of tobacco (*Nicotiana tabacum*) in 0.1 M phosphate buffer (pH 7.2) with 0.4% beta-mercaptoethanol added. Before inoculation with different strains of PVY, plants were tested to ensure their virus free status by ELISA using commercially available kit (Agdia Inc., Elkhart, IN). Four to five leaves of healthy potato plants were dusted with carobrundum powder-320 mesh (Fisher) prior to applying the inoculum using a cotton swab. Following inoculations, each group of plants were kept in separate compartments of a greenhouse with same environmental conditions. In each treatment, ten plants of Russet Burbank were inoculated with each of the three strains of PVY.

SYMPTOMS

Plants were observed for symptom development. Three weeks-post inoculation, two younger, uninoculated leaves were taken from the inoculated plants and tested by DAS-ELISA. Healthy un-inoculated potato samples were included in the ELISA assay as negative controls and only buffer was used as a buffer control. Samples were tested in duplicate wells by using the

equal amount of leaf sample (0.50 gm) in 1 ml of extraction buffer. The plant samples that gave absorbance values three times higher than healthy controls were considered positive to infection with PVY. Leaves from *N. tabacum* plants infected with PVY were included as a positive control.

EXTRACTION OF TOTAL RNA FROM PVY INFECTED AND HEALTHY POTATO PLANTS

From PVY infected plants, total RNA was extracted from PVY-infected potato leaves by using Trizol reagent (Invitrogen) and the quality of RNA was tested by determining the 260/280 and 230/260 ratios of total RNA (Table 1). The integrity of the total RNA was verified on 2% agarose gel (Figure 5).

ANALYSIS OF SMALL RNA SEQUENCES FROM ILLUMINA SEQUENCING DATA

Small RNA reads were quality filtered and reads smaller than 18 nucleotides were removed. Reads were separated according to their lengths after the removal of adaptor tags in silico. The annotation of clean reads to different RNA species through Rfam alignments. The small RNA reads were aligned to known non-coding RNAs (ribosomal RNAs, transfer RNAs, small nuclear RNAs.) obtained from RFAM (http://www.sanger.ac.uk/Software/Rfam/ftp.shtml)

with National Center for Biotechnology Information (NCBI) through BLASTn (Altschul *et al*., 1990). Following the removal of small RNAs corresponding to repeat elements and known non-coding RNAs, unique sequences between 18 and 28 nucleotides were mapped to the recently published complete genomes of different strains of *Potato virus Y* : PVY-O, PVY-N and PVY-NTN (Karasev *et al*., 2011-12 and Lorenzen *et al*., 2005). After mapping to PVY published genomes, the reads were sorted into small RNAs of host and viral origins. The host-derived small RNAs were used to scan miRBase (version 13; http:// microrna.sanger.ac.uk/) and resulted in the identification of conserved miRNA homologues in potato.

IDENTIFICATION OF microRNA FAMILIES AND NOVEL microRNAs

Out of total small RNA sequence reads, microRNAs were sorted out ranging in size from 17-27 nucleotides on the basis of their unique features: their single strandedness, variable 3' and 5' prime ends and due to the presence hairpins. The microRNAs were further classified as conserved and non-conserved by using the miRBase database (GriffithS-Jones *et al*., 2008; Griffiths Jones and Kozomara, 2011) and were assigned into different miRNA families. The microRNAs which were not available in the database were classified as potential novel microRNAs.

RESULTS

SYMPTOMS DEVELOPMENT

Two weeks post-inoculation, inoculated plants started showing symptoms of infection that included systemic mosaic and mottling of leaves. Symptoms became severe three weeks post-inoculation (Figures 2 and 8). Symptoms were visible in case of PVY-O and PVY-NTN

whereas PVY-N did not produce any symptoms in Russet Burbank. Four weeks post-inoculation, PVY-infected plants which showed same virus titers in ELISA were sampled and four-five uninoculated systemic leaves were harvested from each plant infected with different strains of PVY. The harvested leaves were immediately dipped in liquid nitrogen and stored at -80^{0}C for subsequent total RNA extraction.

SMALL RNAs IN *POTATO VIRUS Y* INFECTED POTATO PLANTS

Four cDNA libraries generated from total RNA extracted from PVY- infected and healthy potato leaves were subjected to high throughput sequencing using Illumina sequencing technology. Totals of 12,090,506, 11,268,752, 14,465,392 raw reads were obtained from PVY-O, PVY-NTN and PVY-N infected potato leaves, respectively, whereas from healthy potato a total of 10,259,909 raw reads were obtained. Clean reads were obtained after removal of reads smaller than 18 nucleotides in length; low quality reads and reads without reliable 3 and 5 end adaptors. The sRNAs corresponding to repeat elements and known non-coding RNAs (snRNAs, snoRNAs, tRNAs and rRNAs) were removed. From PVY-infected plants, total clean reads were as follows: from PVY-O infected (12,022,097), PVY-NTN (11,181,527) and from PVY-N infected plants (14,248,648) whereas from healthy potato plant (10,182,597) total clean reads were obtained. High quality reads from 4 libraries were aligned to published genomes of different strains of *Potato virus Y* : PVY-O (GenBank accession number, HQ912895.1), PVY-N (GenBank accession number, AY884983.1) and PVY-NTN (GenBank accession number, JQ924887.1) (Karasev *et al*., 2011-12; Lorenzen *et al*., 2005; Hu *et al*., 2009). Firstly, reads were quality filtered, adaptors were removed and ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) were filtered out from the

data. From potato samples infected with three PVY strains, different RNA species including microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and transfer RNAs were identified through Rfam alignment.

CHARACTERIZATION OF TOTAL SMALL RNAS

For characterizing small RNAs (sRNA), the reads were analyzed with regard to their frequency distribution in PVY-infected and healthy potato samples. Total vsiRNAs were size separated and the population frequency of each size class was determined. As PVY-NTN and PVY-O are severe in potato in comparison to PVY-N, the small RNA profiles of PVY-N showed interesting differences from PVY-NTN and PVY-O. The majority of sRNAs were 21-24 nt in size with the 21 nt being the predominant class in PVY-infected potato samples, whereas in the healthy potato sample, the 24 nt class was found to be the most abundant (Figure 7). The frequency distribution of sRNAs belonging to different size classes was different for each of the PVY strains. The 21 nt class was at the highest level in plants infected with PVY-O (7,934,584), followed by PVY –NTN (7,435,715) and PVY-N (6,819,879). It was interesting to note that except for the 21 nt class, the population of total sRNAs was greater for other size classes in plants infected with (mild) PVY-N compared to the other two strains. The population of 24 nt in PVY-N infected plants was three times higher compared to those infected with PVY-O and PVY-NTN. The 22 nt small RNA profiles were very similar in plants infected with PVY-NTN and PVY-N whereas there were small non-significant differences in the population of 23 nt siRNAs in three PVY strains.

PVY-SPECIFIC SHORT INTERFERING RNAs IN PVY-INFECTED RUSSET BURBANK PLANTS

Total reads identified in PVY-infected plants were mapped to PVY genome and PVY-vsiRNAs were sorted out and the number of reads belonging to each size class was determined.

From each sample, we could identify PVY-vsiRNAs ranging in size from 18 nt to 26 nt representing almost the entire PVY genome. The unique reads mapping to PVY genome in PVY-NTN infected plants were 55,228 which represented 3.13% of total reads and the total population of reads mapping to PVY genome in PVY-NTN infected plants was 4,957,589. In plants infected with PVY-N, there were 57,895 reads that mapped to PVY genome making 1.93% of total reads in PVY-N infected plants. The total population of PVY-vsiRNAs in PVY-N infected plants was 3,887,747. In PVY-O infected plants, reads mapping to PVY genome were 42,680 which represented 1.70 % of total reads and the total population of PVY-vsiRNAs in PVY-O infected plants was 3,347,434. From the above data, it was found that PVY-NTN infected plants accumulated the highest population of PVY-vsiRNAs which was 4,957,589 in comparison to PVY-N (3,887,747 vsiRNAs) and PVY-O (3,347,434 vsiRNAs) infected plants.

In PVY-infected plants, the 21 nt class was the predominant class followed by 22, 20 and 23 nt classes with respect to total vsiRNAs whereas according to unique reads19 and 20 nt classes had more population than 23 nt class (Figure 8A). There were differences in the

population of vsiRNAs belonging to different classes in each of three strains. A considerable difference was found in the population of 21 nt class in PVY-NTN infected plants that had 405 5,439 vsiRNAs in comparison to plants infected with PVY-N and PVY-O: which had 296, 3102 and 2, 859,946 vsiRNAs respectively of 21 nt class. Similarly, the population of 22 nt class was comparatively higher in PVY-NTN infected plants versus PVY-N infected plants: 808,432

vsiRNAs in PVY-NTN infected plants versus 507,769 vsiRNAs in PVY-N infected plants and the population of 22nt class was nearly twice in PVY-NTN infected plants in comparison to PVY-O infected plants: 808,432 vsiRNAs in PVY-NTN infected plants versus 436,313 vsiRNAs in PVY-O infected plants (Figure 8B). The population of total PVY-vsiRNAs belonging to 24 nt class was minimal in PVY-infected plants except for PVY-O infected plants where 24 nt class had more vsiRNAs than 23 nt class; 23,155 vsiRNAs in 24 nt class versus 12, 415 vsiRNAs in 23 nt class. According to unique reads, PVY-N had more visRNAs from 23 nt class: 3,097 of 23 nt class versus 4,140 vsiRNAs of 24 nt class (Figure 8B).

HOTSPOTS FOR VIRUS-DERIVED SHORT INTERFERING RNA(s)

Virus-derived short interfering RNAs (vsiRNAs) of 18-26 nts originated from every genomic position of the PVY genome, however, the frequency and distribution of these vsiRNAs across the PVY genome was not uniform. Certain hotspots were identified which generated more vsiRNAs in comparison to other regions of the genome. Examination of vsiRNA profiles revealed three hotspots in the PVY-N genome both in positive (+) and negative (-) polarities contributing toward the 21 nt vsiRNA class. The first hot spot (HS1) was located in the genomic region from 7300-7800 nts of the large nuclear inclusion protein (NIb) gene, yielding 191,289 vsiRNAs. The HS2 was in the cytoplasmic inclusion protein (C1) gene in the region from 4400-4500 nts which yielded 65,193 vsiRNAs of 21 nt size of both positive (+) and negative (-) polarities. The HS3 was located in the coat protein gene (CP) in the region from 8500-8700 nts from where 56,036 vsiRNAs of 21 nt class were generated (Figure 9A). The vsiRNA profiles indicated two hotspots in PVY-NTN genome which produced vsiRNAs of 21 nt class of both

positive (+) and negative (-) polarities. HS1 was located in the P1 (700-900 nts), generating 289,620 vsiRNAs. HS2 was located in the large nuclear inclusion protein (NIb) in the region of 7750-7800 nts, which produced 66,899 vsiRNAs (Figure 9B). Similarly in the PVY-O genome, vsiRNA profiles revealed two hotspots: HS1 was in the C1 gene (4500-4900 nt) which produced 130,452 vsiRNAs of both negative (+) and positive (-) polarities and the HS2 was identified in NIb in the 200 nt-long stretch between 7400-7600 nt, yielding 155,849 vsiRNAs (Figure 9C).

VIRUS-DERIVED SHORT INTERFERING RNA(s) FROM INDIVIDUAL PVY GENES

Analysis showed that PVY-vsiRNAs originated from every gene of PVY genome. Considerable differences were found in the population of vsiRNAs from different genes of PVY genome. Unique reads as well as total vsiRNAs from each individual gene of PVY were determined (Figures 10 and 11). Some of the genes represented potential sites for vsiRNAs generation. Highest number of unique as well as total vsiRNAs came from cytoplasmic inclusion protein gene (CI) probably due to its larger size (2,000 bp) in PVY-O and PVY-NTN strains whereas from PVY-N, NIb gene produced maximum total as well as unique vsiRNAs. Two other genes: helper component protein gene (Hc-Pro) and first protein gene (P1) also produced highest numbers of vsiRNAs (Figures 10 and 11). Minimum number of vsiRNAs were derived from 6K1 (155bp) and 6K2 (250bp) genes, probably due to their smaller sizes. There was a bias in the generation of vsiRNAs from the sense strand versus antisense strand of PVY genome. From all genes of PVY, number of total sense-derived vsiRNAs was greater than antisense derived vsiRNAs except the P1gene of PVY-NTN which produced more total vsiRNAs from antisense strand: 462,197 from sense versus 425,382 from antisense strand. Similarly, NIb gene in PVY-NTN also produced 238,797 vsiRNAs from the antisense strand versus 41,188 vsiRNAs from the sense strand (Figure 11). In PVY-O, the population of sense versus antisense vsiRNAs from NIb

gene was four times higher: 108,447 reads from the antisense versus 490,278 from the sense strand (Figure 10).

A strong bias was observed in the generation of total vsiRNAs from the sense strand compared to antisense strand. From PVY-NTN, a 1.5:1 ratio of sense to antisense vsiRNAs was observed: 2,924,371 vsiRNAs from sense strand versus 2,033,218 from antisense strand.

Similarly, in PVY-O, there were 2,891,500 vsiRNAs from the sense strand versus 1,590,499 from the antisense, a two times population of sense versus antisense vsiRNAs. PVY-N strain also showed highest population of sense derived vsiRNAs (Figure 10). There was a similar trend for the sense strand-derived vsiRNAs from every individual gene of the PVY genome for all three strains except for the P1 and NIb genes of PVY-NTN strain where the majority of vsiRNAs originated from the antisense strand. Interestingly, it was observed that from the 3 NTR region (325 bp long stretch) antisense vsiRNAs were more in comparison to sense strand derived vsiRNAs in each PVY strain.

NEW AND CONSERVED MICRORNAS IN POTATO CULTIVAR RUSSET BURBANK

We identified 876 unique microRNAs in PVY-N infected potato plants and the total microRNAs population in PVY-N infected plants was 8,596. Likewise, in PVY-NTN infected plants 485 unique microRNAs were identified with an overall population of 2,433. In plants infected with ordinary strain of PVY, there were 755 unique microRNAs with a population frequency of 1,756 whereas in healthy Russet Burbank plants there were 1,266 unique microRNAs and 3,534 total microRNAs. The microRNA profiles in three strains were different indicating that these strains may use different infection mechanism and they interact with the host differently resulting in different host physiological and biochemical reactions.

From the sequenced samples, we identified several conserved and non-conserved microRNAs from 13 microRNA families (Table 6). In addition to conserved microRNAs, 6 novel microRNAs were identified in potato plants infected with three strains of *Potato virus Y* used in the experiment (Table 7).

DISCUSSION

In response to pathogen infection, plants activate RNA silencing mechanism in which pathogen's messenger is targeted and degraded into small siRNA molecules of 21-25 nt. Viruses act as both inducers and targets of host derived RNA silencing, a natural defense mechanism in plants. Both animal and plant viruses induce the generation of 20-25 nucleotide longs siRNAs and miRNAs in infected hosts (Parameswaran *et al*., 2010). These siRNAs and miRNAs restrict virus replication in infected plant cells. Previous studies conducted on small RNA profiles related to plant viruses have been on annual and perennial hosts as well as in experimental indicator hosts (Alabi *et al*., 2012). Information about small RNA profiles of virus infected plants is important to have an insight into virus-host interactions at molecular level. So far, there is no report regarding virus derived small RNAs from PVY- infected potato plants. Our data provides the first report of virus derived small RNAs in potato plants infected with three different strains of PVY: PVY-O, PVY-N and PVY- NTN. This study also provides an insight on the effect of different virus strains on endogenous small RNA regeneration. Populations of viral small RNAs in PVY- infected plants were abundant, diverse and derived from every genomic position of PVY representing that every genomic position could be a putative cleavage site for Dicer- like enzymes. Majority of small RNAs from 21, 22 and 20 nucleotide classes were

derived from the same genomic positions although there were certain hotspots for small RNAs generation. The data revealed that the sRNA populations varied between different strains of PVY in infected Russet Burbank plants. This is probably due to the intrinsic differences in the replication, infection and accumulation mechanisms of different viral strains as well as the host's ability to recognize the different strains and activate gene silencing mechanisms to different strains accordingly. In healthy potato plants, 24 nucleotide class was the predominant class with 48.75% of total small RNAs whereas in PVY-infected plants the 21 nucleotide class was predominant: PVY-O (66.46%), PVY-N (47.50%) and PVY-NTN (66.58%). The second most abundant class in PVY- infected plants was 22 nucleotide size class that constituted 12.84% of siRNAs in PVY-O infected plants, 11.52% in PVY-N infected plants and 15.52% in PVY-NTN infected plants. The majority of 21 and 22 nucleotide class small RNAs also highlights the importance of DCL4 and DCL2 enzymes in the RNA- interference mechanism and nullifying these two enzymes will make plants more susceptible to disease (Ding, 2010 and Llave, 2010). The findings were in accordance with the results of Ding and Voinnet (2007) where they discovered small RNAs in *Arabidopsis* infected with positive stranded RNA viruses belonged to 21 nucleotide class which is produced by DCL3 which in addition to producing 21 nucleotide class siRNAs also generates endogenous trans-acting siRNAs and siRNAs targeting transgenes. Abundance of the 21 and 22 nucleotide classes in virus infected plants is also in accordance with the findings of Lin *et al*., (2010) who analyzed virus derived small RNAs in *Bamboo mosaic virus* infected *Nicotiana Benthamiana* and *Arabidopsis thaliana* plants. They found that the majority of siRNAs were 21 and 22 nucleotides long and the dominance of 21 and 22 nucleotide size siRNAs in virus infected plants has been reported for several other plant viruses and it supports the evidence that the 21 nucleotide long siRNA class is the predominant anti-viral

silencing component. DCL4 is responsible for generation of 21nt-siRNA in virus infected plants (Blevins *et al.*, 2006; Donaire *et al*., 2009, Ho *et al*., 2007, Qi *et al*., 2009, Molnar *et al*., 2005). Another study that was carried out on viruses infecting grapevines by Pantaleo *et al*., (2010), the prevalent vsiRNAs size was 21 nucleotide corresponding to 65% of total vsiRNAs followed by 22 nucleotide species corresponding to 15% of the total vsiRNAs. The RSV siRNA population in infected rice plants was also dominated by species of 21 (44.8%) and 22 nucleotide classes (22.8%) (Yan *et al*., 2010). The above described results indicate that the potato RNA silencing machinery responsible for the biogenesis of endogenous small RNAs tends to produce 24 nucleotide class small RNAs as long in the absensce of invading pathogen. The maximum population of small RNAs was found in PVY-NTN infected plants (4,957,589) followed by PVY-N (3,887,747) and PVY-O (3,347,434). A high population of small RNAs (20-24 nt) in potato plants infected with PVY-N and PVY-NTN correlates with the fact that these strains are more virulent in Russet Burbank. Ordinary strain produces systemic mosaic and mottling in Russet Burbank plants whereas PVY-NTN induces chlorotic mosaic and brown spots on tubers. In our findings, we observed that PVY-O infected potato plants had comparatively less population of siRNAs than the plants infected with PVY-NTN and PVY-N. There could be two possible reasons for the differences in the accumulation of virus derived siRNAs in PVY-infected plants. It could be that plants developed a stronger RNA silencing mechanism towards necrotic strains (N and NTN) which resulted in an increased level of RNA interference to counteract the aggressive pathogen whereas in case of ordinary strain (PVY-O) the plants showed a weaker gene silencing response, which subsequently resulted in comparatively less siRNAs in PVY-O infected Russet Burbank plants.

Unique vsiRNAs from individual PVY strains represented a genome wide distribution and every genome position was occupied by at least one vsRNA. Our results also demonstrated that vsRNAs were oriented towards positive sense of the genome in comparison to the negative sense strand. The findings are in accordance with the recent findings of Ho *et al*., (2007) and Qi *et al*., (2009) who reported more siRNAs were derived from plus strand in comparison to the minus strand of genomes of *Turnip mosaic virus* (TuMV) and *Tomato mosaic virus* (TMV). But the results were not in accordance with the findings of Silva *et al.,* (2011), where they reported an equal population of sense versus antisense derived virus small RNAs in *Cotton leafroll dwarf virus* infected cotton plants. Derivation of sense vsRNAs from perfectly complementary dsRNA derived from intermolceular base pairing of positive and negative viral strands is relatively easy to understand. Accumulation of positive sense siRNAs supports a model by which folded RNA within viral ssRNA serves as a substrate for DCL cleavage (Molnar *et al*., 2005). There was the same kind of association of sense versus antisense derived siRNAs from different individual genes of PVY- genome except for the P1 gene of PVY- NTN strain where most siRNAs came from the antisense strand of the genome (Table 6). The findings also contrasted with the findings of Li *et al*., (2012) where majority of siRNAs were of (-) polarity than those in (+) polarity for *Potato spindle tuber viroid* (PSTVd) in tomato CAHN8. In fact, nearly two thirds (63.6%) of PSTVd siRNAs were in the (-) polarity, with only one third (36.4%) in (+) polarity, Interestingly, in the case of PepMV, the (-) polarity siRNAs were also prevalent over the (+) polarity. The findings were in accordance with the findings of Kreuze *et al*., (2004) where they reported the prevalence of positive sense small RNAs over negative sense in virus infected sweet potato plants. The data showed that Dicer enzymes are biased in their target cleavage towards the positive- sense RNA strands in comparison to the negative sense strands that resulted in high

population of plus-sense derived siRNAs. This could be due to evolution or as a result of arms race between pathogen and host. As majority of plant infecting viruses are positive sense single-stranded viruses so being biased towards the positive sense of the genome will give the host an advantage to defeat the virus at an early step of their life cycle and stop transcription of virus genome. Examination of the relative abundance of sense and antisense viral small RNAs from the different RNA fragments of PVY genome showed interesting results (Figure 7). Only in case of NIb gene of PVY-NTN strain, the antisense derived small RNAs were more than the sense strand. For all other genes of PVY genome, the number of sense derived small RNAs was greater than antisense derived small RNAs. Hotspots of vsiRNAs accumulation are represented by sharp and broad peaks of vsiRNA abundance scattered throughout the viral genome. These peaks are clusters of multiple reads representing several unique vsiRNA sequences; sharp peaks denote the presence of highly abundant reads within the cluster. The pattern of hotspots in plants infected with different PVY strains indicated differences in the presence of hotspots (Figure 6). The hotspots were distributed throughout the viral gene fragments. The findings were in accordance with the findings of Donaire *et al*., (2009) who found certain hotspots in viral genomes. This showed that in case of viral genomes there is tendency for certain regions to produce more siRNAs in comparsion to other regions of the genome. The location of distinct hotspots indicates that Dicer enzymes have more target affinity to these regions of viral genomes. The origin of siRNAs preferentially from certain regions of the gnome rises many possibilities; either these regions have more tendency to foldback into hairpin precursors or they have some role in viral replication cycle. In my data, I found that NIb gene that acts as polymerase in the viral life cycle, produced maximum siRNAs had hotspots in it. Possibly, the polymerase gene is main target for endonuclease enzymes in comparsion to other viral genes. Hotspots were also located in the

Protease 1 (P1) gene that has a very important role in the virus life cycle. The preferrenial accumulation of positive sense siRNAs is a general characteristic found in positive sense RNA viruses (Molnar *et al*., 2005). In addition to vsiRNAs, we also analyzed microRNAs in PVY-infected as well as healthy potato plants. There were considerable differences in the population of unique nicroRNAs in potato plants infected with three different PVY-strains. These results suggested that miRNAs are being regulated with the virus strain infecting the plants. As these microRNAs have certain roles in plant growth and development as well as defense against invading pathogens and viruses, the production of miRNAs is also being controlled by the different PVY strains. All the identified microRNAs belonged to 13 different miRNA families and were expressed differently for different strains.

The study has reported the small RNA profiles of three distinct strains of PVY as well as novel and candidate microRNAs from potato genome. The strainal variation among the siRNA profiles, host derived endogenous small RNAs are provide important clues to understanding the infection mechanism and pathogenicity of this important virus of potato. As PVY is commonly detected in mixed infections of multiple PVY strains and in mixed infection with other potato viruses, the findings will make it easier to differentiate between multiple viruses infecting same plant through the use of deep sequencing technology. This is the first report of the siRNA profiles of PVY infection of a popular potato cultivar. The identification of novel and conserved miRNAs from potato genome will increase the reportier in the miRBase (Griffith-Jones *et al*., 2007). Our results can be useful in designing antiviral strategies using RNAi against potyviruses and further understating of symptom expression and silencing suppression with different strains of potyviruses.

REFERENCES:

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Bazzini AA., Hopp HE., Beachy RN. and Asurmendi S. (2007). Infection and co-accumulation of *Tobacco mosaic virus* proteins alter microRNA levels, correlating with symptom and plant development. Proceedings of the National Academy of Sciences, USA, 104: 12 157–12162.

Beczner L, Horvath J, Romhanyi I, Foorster H. (1984). Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Research 27: 339–343.

Bi Y. Tugume AK, and Valkonen JPT. (2012) Small RNA deep sequencing reveals *Arctium tomentosum* as a natural host of *Alstroemeria virus X* and a new putative Emaravirus. PLOS ONE. 7(8). e42758

Blevins T, Rajeswaran R, Shivaprasad PV, Beknazariants D, Si-Ammour A, Park HS, Vazquez F, Robertson D, Meins JF, Hohn T, Pooggin MM. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Research. 34: 6233-6246.

Brodersen P and Voinnet O. (2006). The diversity of RNA silencing pathways in Plants. Trends in Genetics. 22: 268-280.

Cillo, F., Mascia, T., Pasciuto, MM. and Gallitelli D. (2009). Differential effects of mild and severe Cucumber mosaic virus strains in the perturbation of microRNA regulated gene expression in tomato map to the 3′ sequence of RNA 2. Molecular Plant–Microbe Interactions. 22: 1239– 1249.

De Bokx JA. (1961). Waard planten van het aardappel- YN-virus. T Pl-ziekten 67: 333–342.

Di SF, Gisel A, Navarro B, Delgado S, Martinez Alba AE. (2009). Deep Sequencing of the Small RNAs Derived from Two Symptomatic Variants of a Chloroplastic Viroid: Implications for Their Genesis and for Pathogenesis. PLoS ONE 4: e7539.

Ding SW. 2010. RNA-based antiviral immunity. National Rev. Immunology. 10: 632–644.

Ding SW and Voinnet O. (2007). Antiviral immunity directed by small RNAs. Cell 130: 413–426.

Donaire L, Wang Y, Gonzalez-Ibeas D, Mayer KF, Aranda MA, Llcave C. (2009). Deep sequencing of plant viral small RNAs reveals effective and widespread targeting of viral genomes. Virology 392: 203-214.

Dougherty WG and Carrington JC. (1988). Expression and function of Potyviral gene products. Annual Review of Phytopathology. 26: 123-143.

Dunoyer P. and Voinnet O. (2005). The complex interplay between plant viruses and host RNA-silencing pathways. Current Opinion in Plant Biology. 8: 415–423.

Edwardson JR (1947). Some properties of the potato virus group. Florida Agricultural Experiment Stations Monograph Series.4: 398.

Ellis P., Stace-Smith R. and de Villiers G. (1997). Identification and geographic distribution of serotypes of potato virus Y. Plant Disease. 81: 481-484.

Fauquet CM, Mayo MA, Maniloff J, Dessel berger U, Ball LA. (2005). Virus taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA, USA, 1259 pp

Galvino-Costa SBF, Figueira AR., Rabelo-Filho FAC, Moraes FHR., Nikolaeva OV and Karasev AV. (2012). Molecular typing of *Potato virus Y* isolates from Brazil reveals a diverse set of recombinant strains. Phytopathology 101: S57.

Gonzalez-Jara P, Tenllado F, Martinez-Garcia B, Atencio FA, Barajas D, Vargas M, Diaz-Ruiz J, and Diaz-Ruiz JR. (2004). Host-dependent differences during synergistic infection by *Potyviruses* with *Potato virus X.* Molecular Plant Pathology. 51: 29-35.

Griffiths-Jones S., Saini HK., Van Dongen S., and Enright, AJ. (2008). "MiRBase: tools for microRNA genomics". Nucleic Acids Research 36: D154–8.

Grundhoff A. and Sullivan, CS. (2011). Virus encoded miRNAs. Virology, 411: 325–343.

He L, Hannon GJ. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nature 5 : 522–531.

Heller, R.A. Schena M, Chai A, Shalon D, Bedilion T, Gilmore J, Woolley DE, and Dawis RW. (1997). Discovery and analysis of inflammatory disease−related genes using cDNA microarrays. Proc. Natl Acad. Sci. USA 94: 2150−2155.

He, XF., Fang, YY., Feng, L. and Guo, HS. (2008). Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR-NBS-LRR class R gene-derived novel miRNA in Brassica. FEBS Lett. 582: 2445–2452.

Hijmans R. (2001). Global distribution of the potato crop. American Journal of Potato Research. 78: 403-12.

(http://www.potatovirus.com/images/etc/gallery/YukonGoldTuber_NTN_gallery.773.jpg). (Date retrieved 11/15/2013)

(http://www.plantwise.org/Uploads/CompendiaImages/Normal/pvy__01.jpg). (Date retrieved 11/15/2013)

(http://www.potatovirus.com/images/etc/gallery/AtlanticN-Wi4_gallery.2606.jpg). (Date retrieved 11/15/2013)

Ho T, Wang H, Pallet D, Dalmay T. (2007). Evidence for targeting common siRNA hotspots and GC preference by plant Dicer-like proteins. FEBS Lett 581: 3267-3272.

Huang J, Hao P, Chen H, Hu W, Yan Q, Liu F and Han ZG. (2009). Genome-wide identification of Schistosoma japonicum miRNAs using a deep-sequencing approach. PLoS ONE 4(12): e8206. doi:10.1371/journal.pone.0008206.

Jingyu Z, Yunyuan X, Qing H, and Kang C. (2009). Deep sequencing of Brachypodium small RNAs at the global genome level identifies microRNAs involved in cold stress response. BMC Genomics. 10: p449.

Jones, RAC. (1990). Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. Annals of Applied Biology 117: 93–105.

Karasev, AV., Meacham, T., Hu, X., Whitworth, JL., Gray, SM., Olsen, N., Nolte, P. (2008). Identification of Potato virus Y Strains Associated with Tuber Damage during a Recent Virus Outbreak in Potato in Idaho. Plant Disease. 92: 1371.

Koch K. (1931). The potato rugose mosaic complex. Science 73: 615.91.

Kreuze JF, Perez A, Untiveros M, Quispe D, Fuentes S. (2009). Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. Virology 388: 1–7.

Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 21: 4663–4670.

Lin, KY, Cheng CP, Wang WC, Huang YW, Lee YS, Huang HD, Hsu YH, Lin NS. (2010). Global analysis of small interfering RNAs derived from Bamboo mosaic virus and its associated satellite RNAs in different plants. PloS ONE 5: e11928.

Li R, Gao S, Hernandez AG, Wechter WP and Fei Z. (2012). Deep sequencing of small RNAs in tomato for virus and viroid identification and strain differentiation. PLoS ONE 7(5): e37127.

Llave C. (2010). Virus-derived small interfering RNAs at the core of plant-virus interactions. Trends in Plant Science. 15: 701–707.

Lorenzen JH., Meacham T., Berger PH., Shiel PJ., Crosslin JM., Hamm PB and Kopp H . (2006). Whole genome characterization of *Potato virus Y* isolates collected in the western USA and their comparison to isolates from Europe and Canada. Archives of Virology. 151: 1055-1074.

Mendez-Lozano, J., Torres-Pacheco, I., Fauquet, CM., and Rivera-Bustamante, RF. (2003). Interactions between geminiviruses in a naturally occurring mixture: Pepper huasteco virus and Pepper golden mosaic virus. Phytopathology 93: 270-277.

Molina LG, Fonseca GC, Morais GL, Oliveira LFV, Carvalho JB, Kulcheski FR, and Margis R. (2012). Metatranscriptomic analysis of small RNAs present in soybean deep sequencing libraries. Genetics and Molceular Biology. 35 (1): 292-303.

Molnar A, Csorba T, Lakatos L, Varallyay E, Lacomme C and Burgyan J. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. Journal of Virology. 79: 7812-7818.

Navarro B, Pantaleo V, Gisel A, Moxon S, Dalmay T, Bisztray G, Serio FD, Burgyan J. (2009). Deep sequencing of the viroid derived small RNAs from grapevine provides new insight on the role of RNA silencing in plant-viroid interaction. PLoS ONE 4(11): e7686. doi:10.1371/journal.pone.0007686.

Nie X, Singh RP, Singh M. (2004). Molecular and pathological characterization of N: O isolates of the Potato virus Y from Manitoba, Canada. Canadian Journal of Plant Pathology. 26: 573–583.

Nykanen A, Haley B, and Zamore PD. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107: 309-321.

Pantaleo V, Salderelli P, Miozzi L, Giampetruzzi A, Gisel A, Moxon S, Dalmay T, Bisztray G, Burgyan J. (2010). Deep sequencing analysis of viral short RNAs from a Pinot Noir infected grapevine. Virology 408: 49-56.

Qi X, Bao FS, Xie Z. (2009). Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA- dependent RNA polymerases in viral siRNA biogenesis. PLoS ONE. 4: 4971.

Quanjer HM (1924) Standardizing of degeneration diseases of potato. Phytopathology 14: 518–520.

Qu F. (2012). Antiviral role of plant encoded RNA-dependent RNA polymerases revisited with deep sequencing of small interfering RNAs of virus origin. Molecular Plant-Microbe interactions. 23: 1248-1252.

Rochow, WF., and Ross, A F. (1955). Virus multiplication in plants doubly infected by potato viruses X and Y. Virology 1: 10-27.

Ronaghi M, Karamohamed S. Pettersson B, Uhlen M and Nyren P. (1996). Real-time DNA sequencing using detection of pyrophosphate release. Analytical Biochemistry. 242: 84-89.

Ronaghi M (2001). Pyrosequencing sheds light on DNA sequencing. Genome Research. 11: 3-11.

Silva TF, Romanel EAC, Andrade RRS, Farinelli L, Osteras M, Deluen C, Correa RL, Schrago CEG, and Vaslin MFS. (2011). Profile of small interfering RNAs from cotton plants infected with the polerovirus *Cotton leafroll dwarf virus*. Molecular Biology, 12: 40.

Simon M, Runchun J, Gyorgy S, Frank S, Rachel LRP, Vincent M. and Tamas D. (2008). Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genome Research. 18: 1602-1609.

Simon-Mateo, C. and Garcia, JA. (2011). Antiviral strategies in plants based on RNA silencing. Biochim. Biophys. Acta, 1809: 722–731.

Singh, RP., Valkonen JP, Gray SM, Boonham N, Jones RA, Kerlan C, and J. Schubert. (2008). Discussion paper: The naming of *Potato virus Y* strains infecting potato. Archives of Virology 153: 1–13.

Smith KM. (1931). Composite nature of certain potato viruses of the mosaic group. Nature 127: 702.

Smith AM, Heisler ME, St Onge RP, Wallace IM, Bodeau J, Harris AN, Farias-Hesson E *et al*. 2010. Highly multiplexed barcode sequencing: an efficient method for parallel analysis of pooled samples. Nucleic Acids Research. 38 (13): e142.

Sunkar R., Li YF and Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. Trends Plant Sci. 17: 196–203.

Syller J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. Molecular Plant Pathology. 13(4): 204-216.

Tatineni S, Graybosch RA, Hein GL., Wegulo SN, and French R. (2009). Wheat cultivar specific disease synergism and alteration of virus accumulation during co-infection with *Wheat streak mosaic virus* and *Triticum mosaic virus*. Virology. 230-238.

The Potato Genome Sequencing Consortium, (2011). Genome sequence and analysis of the tuber crop potato. 475: 189-195.

Torrance, L., IA. Andreev, R. Gabrenaite-Verhovskaya, G. Cowan, K. Makinen, and ME. Taliansky. (2006). An unusual structure at one end of potato potyvirus particles. Journal of Molecular Biology 357: 1–8.

Valleau WD, Johnson EM. (1930). The relation of some tobacco viruses to potato degeneration. Kentucky Agr. Exp. Sta. Bul. 309: 475–507.

Valouev A, Ichikawa J, Tonthat T, Stuart J, Ranade S, Peckham H, Zeng K *et al*., 2008. A high resolution nucleosome position map of C. elegans reveals a lack of universal sequence dictated positioning. Genome Research. 18(7): 1051-1063.

Vance, VB. (1991). Replication of potato virus X RNA is altered in coinfection with potato virus Y. Virology 182: 486-494.

Vijayapalani P, Maeshima M, Nagasaki-Takekuchi N and Miller A. (2012). Interaction of the trans-frame potyvirus protein P3N-PIPO with host protein PCaP1 facilitates potyvirus movement. PloS Pathogens. 8(4): e1002639.

Vitantonio P, Pasquale S, Laura M, Annalisa G, Andreas G, Simon M, Tamas D, Gyorgy B, and Jozsef B. (2010). Deep sequencing analysis of viral short RNAs from an infected Pinot Noir grapevine. Virology, 408: 49-56.

Ward CW and Shukla DD (1991). Taxonomy of Potyviruses. Current problems and possible solutions. Intervirology 32: 269-296.

Wang XB., Wu Q., Ito T., Cillo F., Li WX., Chen X., Yu JL. and Ding SW. (2010). RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA, 107: 484–489.

Wei B, Cai T, Zhang R, Li A, Hiu N, Li S, Gu YQ, Vogel J, Jia J, Qi Y and Mao L. (2009). Novel miRNAs uncovered by deep sequencing of small RNA trasnscriptomes in bread wheat (*Triticum aestivum* L.) and *Brachypodium distachyon* (L.) Beauv. Functional and Integrative Genomics, 9: 499-511.

Wintermantel, WM., Cortez, AA., Anchieta, AG., Gulati-Sakhuja, A.,and Hladky, LL. (2008). Co-infection by two criniviruses alters accumulation of each virus in a host-specific manner and influences efficiency of virus transmission. Phytopathology 98:1340-1345.

Wu Q, Luo Y, Lu R, Lau N, Lai EC, Li WX, and Ding SW. (2009). Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. PNAS. 107: 1606-1611.

Xu Y, Huang L, Fu S, Wu G and Zhou X. (2012). Population diversity of rice stripe virus derived siRNAs in three different hosts and RNAi based antiviral immunity in *Laodelphgax striatellus.* PLoS ONE. 7: e46238.Yang X, Yang Y, Guo W, Xie Y and Xie Q. (2011). Characterization of small interfering RNA derived from the geminivirus / betasatellite complex using deep sequencing. PLoS ONE 6: e16928.

Yan F, Zhang H, Adams MJ, Yang J, Peng J, Antoniw JF, Zhou Y, and Chen J. (2010). Characterization of siRNAs derived from rice stripe virus in infected rice plants by deep sequencing. Archives of Virology. 155(6): 935-40.

Zhao CZ, Xia H, Frazier TP, Yao YY, Bi YP, Li AQ, Li MJ, Li CS, Zhang BH, and Wang XJ. (2010). Deep sequencing identifies novel and conserved microRNAs in peanuts (*Arachis hypogaea* L.). BMC Plant Biology. 1471-2229/10/3

Table 1: Quality analysis of total RNA extracted from potato plants infected with different strains of Potato virus Y (PVY): ordinary strain (PVY-O), tobacco veinal necrotic strain (PVY-N) and tuber necrosis strain (PVY-NTN).

Sample Name	*260/280 ratio*	*260/230 ratio*
RNA from PVY-O infected Russet Burbank	2.18	2.57
RNA from PVY-N infected Russet Burbank	2.03	1.77
RNA from PVY-NTN infected Russet Burbank	2.03	2.09
RNA from healthy Russet Burbank leaves	1.84	1.09

Table 2: *Potato virus Y*-ordinary strain (PVY-O) small RNAs in infected Russet Burbank leaves

Category of Small RNA	**Reads**
Total raw reads	12090506
High quality	12053782
Smaller than 18 nt	13120
Clean reads	12022097
Short interfering RNAs mapped to PVY genome	42680
Unique miRNAs	755
Total miRNAs	1756
Small nuclear RNAs	2358
Reads not mapping to host or pathogen genome	11755213

Table 3: *Potato virus Y*-necrosis tuber-necrotic strain (PVY-NTN) small RNAs in infected Russet Burbank leaves

Category of Small RNA	**Reads**
Total Raw reads	11268752
High quality	11232128
Smaller than 18 nt	38227
Clean reads	11181527
Short interfering RNAs mapped to PVY genome	55228
Unique miRNAs	485
Total miRNAs	2433
Small nuclear RNAs	2442
Reads not mapping to host or pathogen genome	10969279

Table 4: *Potato virus Y*- tobacco veinal necrotic strain (PVY-N) small RNAs in infected Russet Burbank leaves

Category of Small RNA	**Reads**
Total Raw Reads	14465392
High Quality	14357640
Smaller than 18 nt	77946
Clean Reads	14248648
Short interfering RNAs mapped to PVY genome	57895
Unique microRNAs	876
Total microRNAs	8596
Small nuclear RNAs	2552
Reads not mapping to host or pathogen genome	14026091

Table 5: Small RNAs from leaves of healthy potato plants

Category of Small RNA	**Reads**
Total Raw Reads	10259909
High Quality	10224605
Clean Reads	323
Short interfering RNAs mapped to PVY genome	1266
Unique microRNAs	3534
Total microRNAs	2516
Small nuclear RNAs	12429
Reads not mapping to host or pathogen genome	9871069

Table 6: Unique sense and antisense derived small RNA counts of *Potato virus Y*- in potato plants infected with *Potato virus Y*-ordinary strain (PVY-O), *Potato virus Y*-tobacco veinal necrotic strain (PVY-N) and *Potato virus Y*-necrosis tuber necrotic strain (PVY-NTN).

		5NTR	P1	Hc-Pro	P3	6K1	CI	6K3	VPg	NIa	NIb	CP	3NTR
PVY-O	Plus	492	2107	3709	2443	234	4376	483	1411	1869	4000	1824	375
	Minus	475	1721	2789	2096	224	4263	274	1055	1573	3066	1547	613
PVY-N	Plus	562	2816	4938	3406	595	5676	507	1806	2544	5362	2931	948
	Plus	528	2300	4100	3018	439	4683	301	1371	1969	3928	2264	874
PVY-NTN	Plus	383	2924	4578	3633	394	5369	385	1566	2013	4758	2464	722
	Minus	328	2683	4117	3351	397	5407	276	1336	1706	3990	2083	788

Table 7: Sense and antisense total virus-derived short-interfering RNAs (vsiRNAs) from potato plants infected with ordinary strain (PVY-O), tobacco veinal necrotic strain (PVY-N) and necrosis tuber necrotic strain (PVY-NTN) of *Potato virus Y*.

PVY strain	Strand	P1	Hc-Pro	P3	6K1	CI	6K2	VPg	NIa	NIb	CP	3NTR
PVY-O	Antisense	154172	186910	83978	6859	335334	6260	58834	78435	108447	66648	34258
	Sense	305095	228891	224367	13814	379175	15925	136285	191659	490278	154466	12104
PVY-N	Antisense	142582	198604	171175	38708	228562	6956	53977	61982	258171	96686	44411
	Sense	262134	478612	191039	62849	366267	38456	141224	196902	496647	265776	34789
PVY-NTN	Antisense	462197	312135	219347	1408	504817	6121	64865	60714	238797	117055	35882
	Sense	425382	542776	462977	51	519977	27360	108267	137451	41188	219039	17394

Table 8: MicroRNA families from *Potato virus Y*- infected and healthy potato plants

miRNA family	Count	Size	Sequence
miR-M1-4-5p	660	24	TCGACAGCACGTGTGTATTCT
miR-M1-13-3p	66	24	ATCTCATGTAGAGGCACTTACTTT
miR-H4-5p	848	24	AGTGAGATTTGAACAGGCAAGCAC
miR-B20	726	23	CGAGTGAGAGTGGCGAGCGAGAT
miR-M1-1-5p	121	22	AGAGAAGTGGGCTCCAATTCTT
miR-J1-5p	36	24	TTTTAGACTGACTGGGAAAAGCAT
miR-M26-5p	6077	21	TCCTTTGAGTTGTGTGGATGA
miR-H21	62	21	ATTAGGTCAGCTGTCTCACGG
miR-H14-3P	172	23	AGCTACATTGTCTGCTGGGTTTC
miR-m59-2	628	23	CCCGAATGAGCCCTGCAAGAGCG
miR-M1-4-5p	1040	24	AGTGAGATTTGAACAGGCAAGCAC
miR-I1-3p	834	22	ATATTCCTCTCTTTCTCTCTCC
miR-H1-5p	35	24	ATGGAAGGACGTGGTGTAAGTGGA

Table 9: Novel MicroRNAs in *Potato virus Y*- infected potato plants

miRNA	Sequence(5-3')	Number of reads
RB N-m0001-3p	GTAGCAGTGACTATGTCTGGA	2332
RBN-m0001_3p	TGGGTCCACAATATCACCTTT	59
RBO-m0002_3p	GTAGCAGTGACTATGTCTGGA	1897
RBNTN-m0001_5p	AGGGGAGCTGTTGGGTCTGGA	265
RBNTN-m0002_5p	TCTTCAGGCCTTTGATGGATG	41
RBNTN-m0003_3p	AGGTGATATTGTGGACCCAAG	2999

FIGURE LEGENDS

Figure 1: Schematic illustration of Sanger sequencing

Figure 2: Schematic illustration of 454-Pyrosequencing

Figure 3: Schematic illustration of Illumina Sequencing

Figure 4: Schematics of Bionformatics work done on small RNA reads. Boxed with cross indicate work that was not done whereas box with star mark shows work done by Khalid Naveed

Figure 5: Agarose gel electrophoresis of total RNA from *Potato virus Y*- infected potato leaves. RNA run as control on gel (Lane1). Total RNA from Russet Burbank infected with *Potato virus Y*- ordinary strain (PVY-O) (Lane 2). Total RNA from healthy Russet Burbank leaves (Lane 3). Total RNA extracted from Russet Burbank leaves infected with *Potato virus Y*- tobacco veinal necrotic strain (PVY-N) (Lane 4). Total RNA extracted from Russet Burbank plants infected with *Potato virus Y*- necrosis tuber-necrotic strain (PVY-NTN) (Lane 5).

Figure 6: Distribution frequency of total small RNAs in *Potato virus Y*- infected potato plants: Total small RNAs with regard to their size distribution in the healthy potato infected with ordinary strain (PVY-O), potato infected with tobacco veinal necrotic strain (PVY-N) and potoato infected with necrosis tuber- necrotic strain (PVY-NTN).

Figure 7: Distribution and frequency of unique and total vsiRNAs in *Potato virus Y* (PVY) - infected Russet Burbank plants. (A) Comparison of unique vsiRNAs in potato plants infected with three PVY strains: PVY-N, PVY-O and PVY-NTN. (B) Total vsiRNAs in potato plants infected with PVY-N, PVY-O and PVY-NTN.

Figure 8: Graphical representation of small RNA reads from sense versus antisense strands of *Potato virus Y* (PVY) genome: (A) Small RNA reads along with genomic positions from *Potato virus Y*- ordinary strain in infected Russet Burbank plants, (B) Small RNA reads along with genomic position from *Potato virus Y*- necrosis tuber necrotic strain (PVY-NTN) in infected Russet Burbank plants, (C) Small RNA reads along with positions in the genome from *Potato virus Y*- tobacco veinal necrotic strain (PVY-N) in infected Russet Burbank plants.

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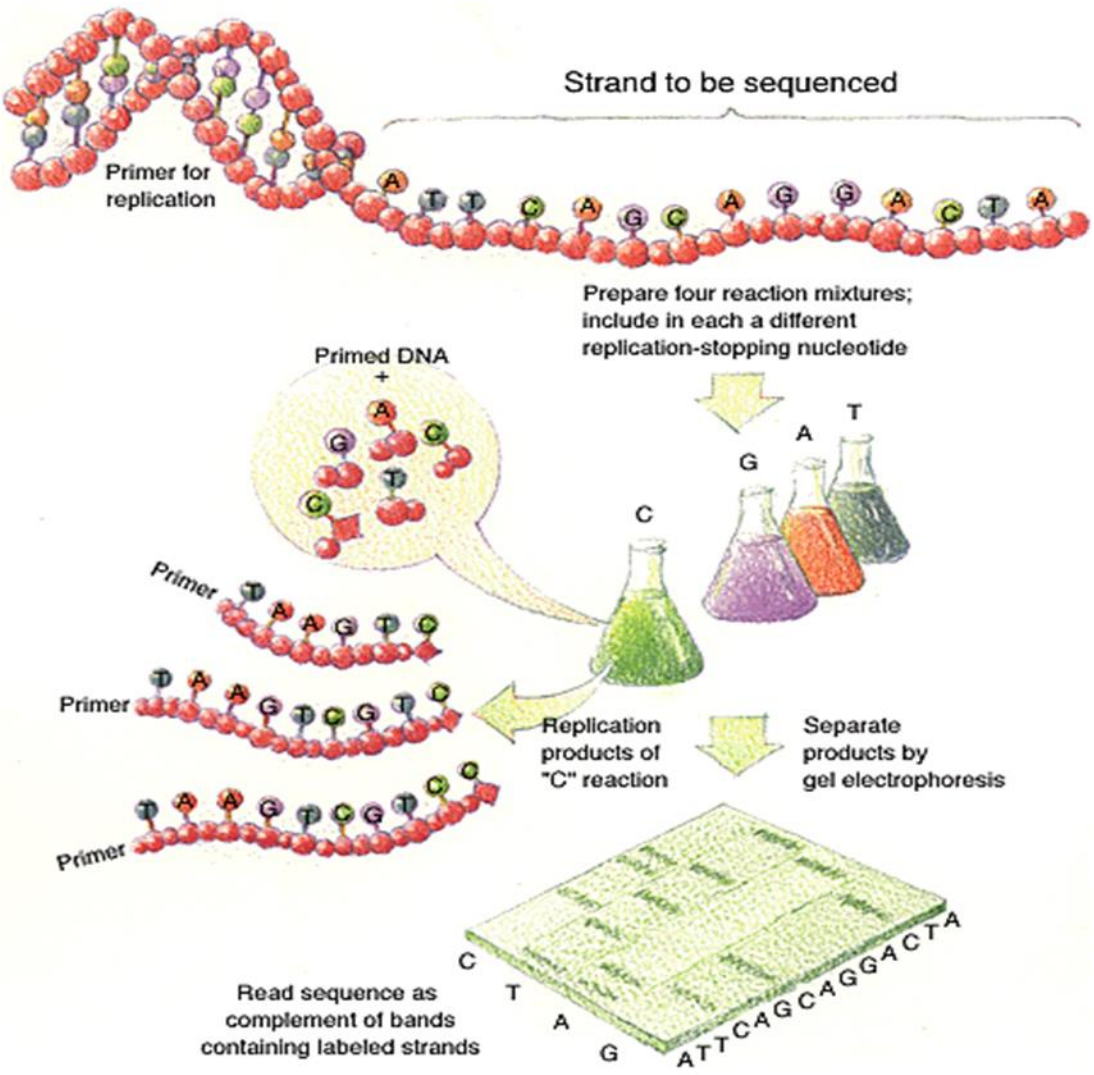

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Figure 1: Schematic illustration of Sanger sequencing

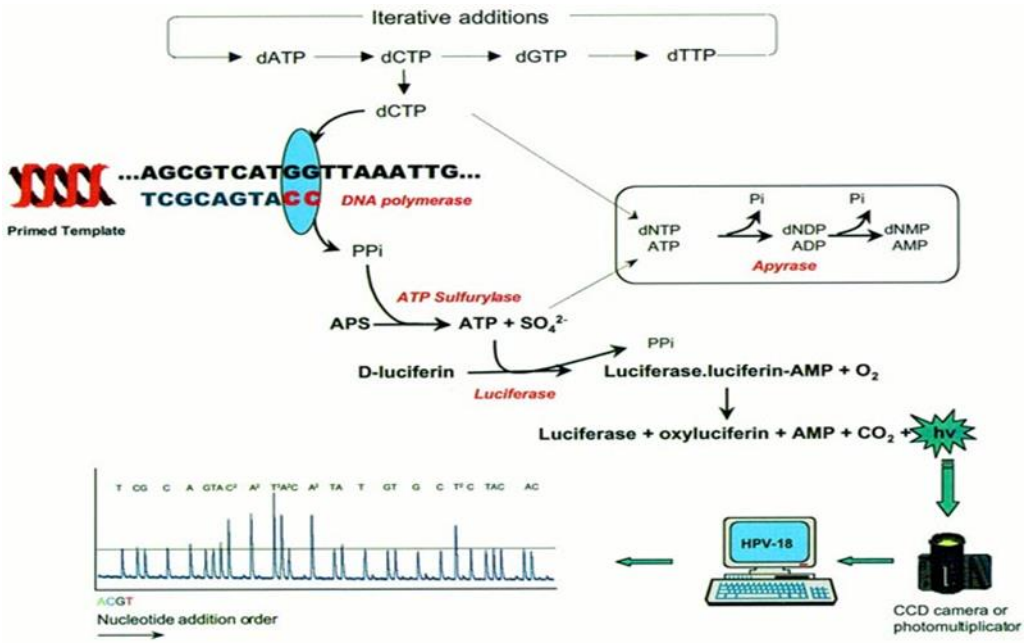

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Figure 2: Schematic illustration of 454-pyrosequencing.

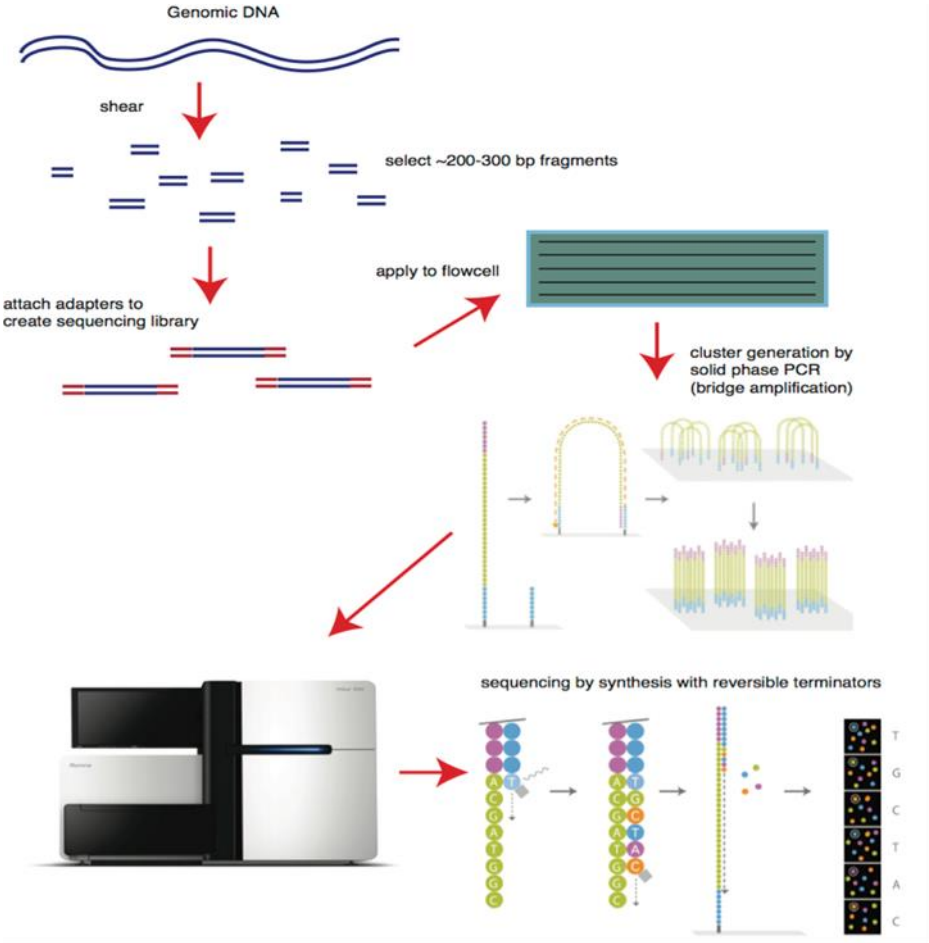

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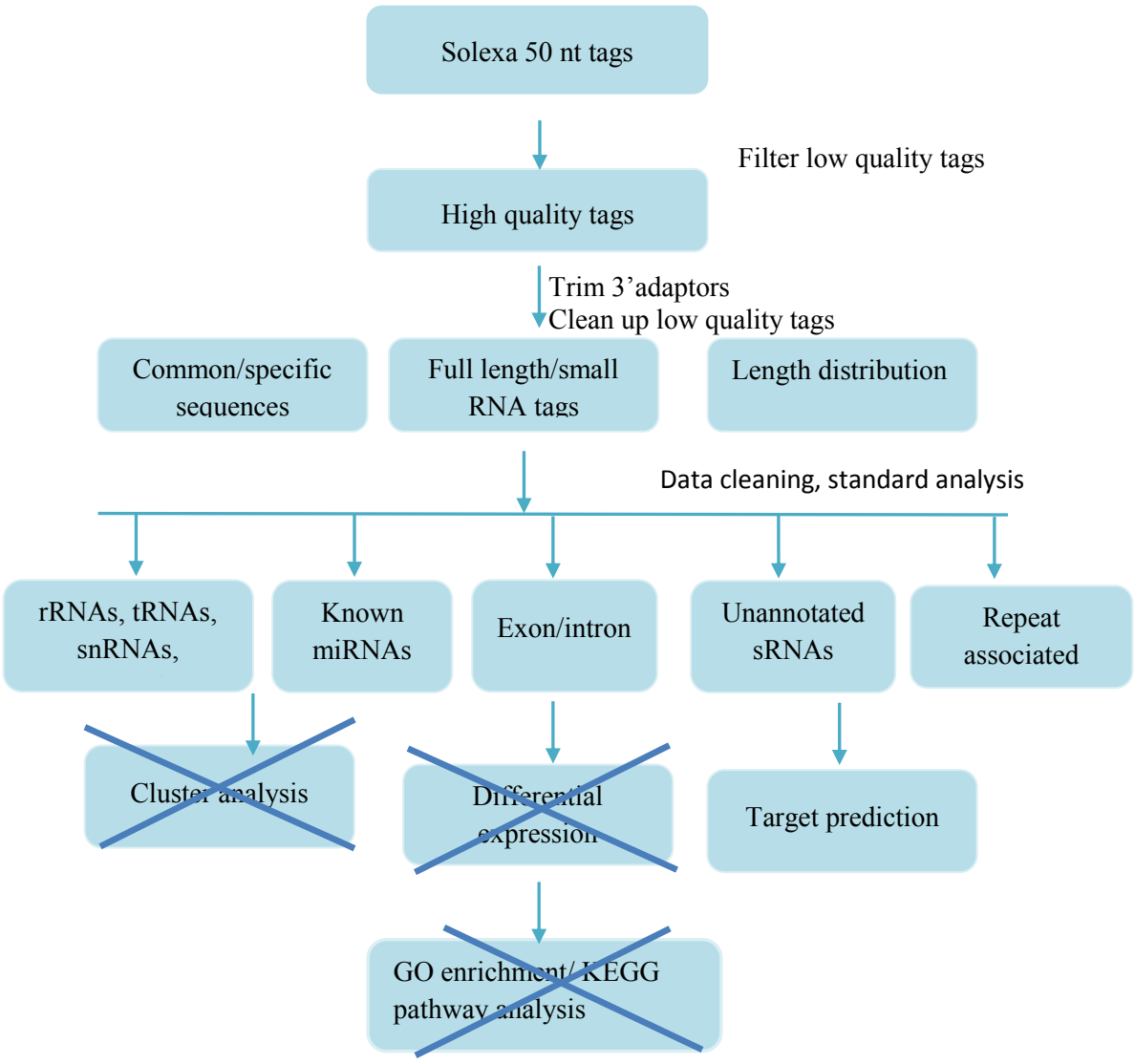
Figure 3: Schematic representation of Illumina sequencing



(Beijing Genomics Institute, Hong Kong)

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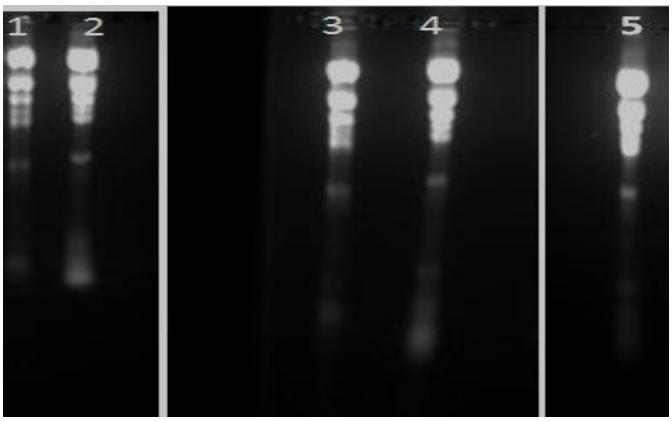

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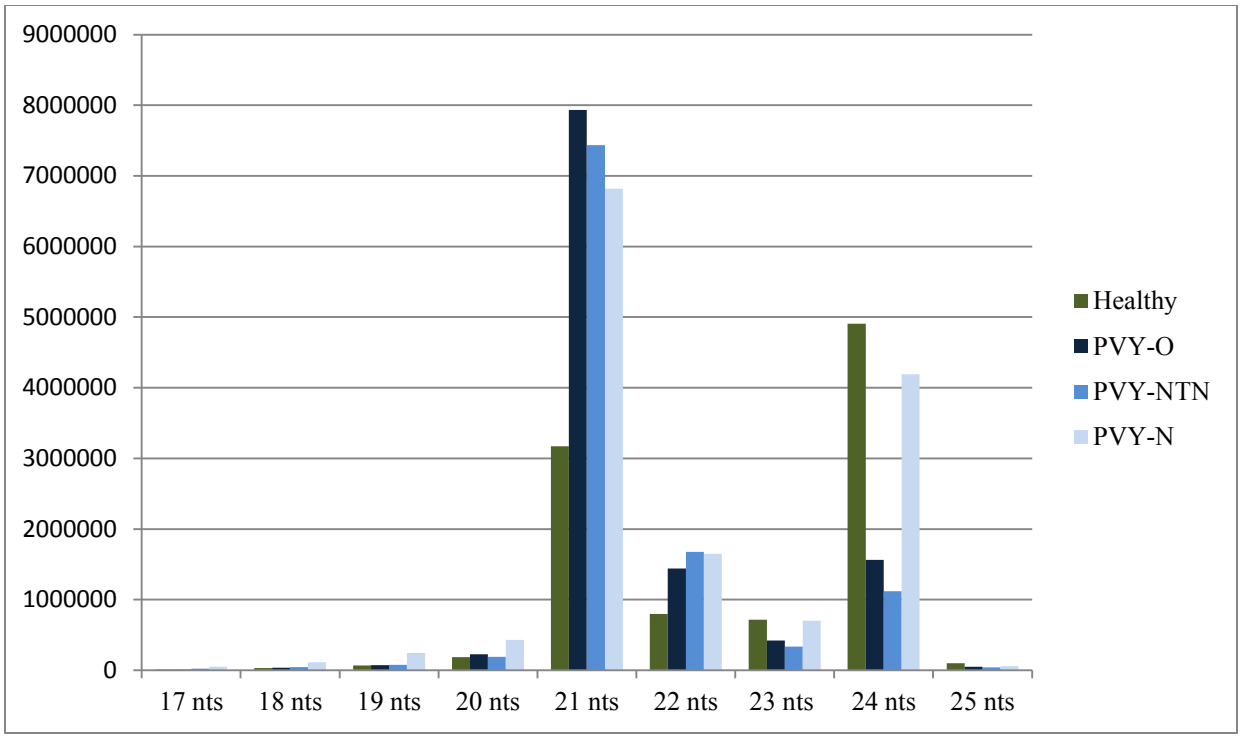

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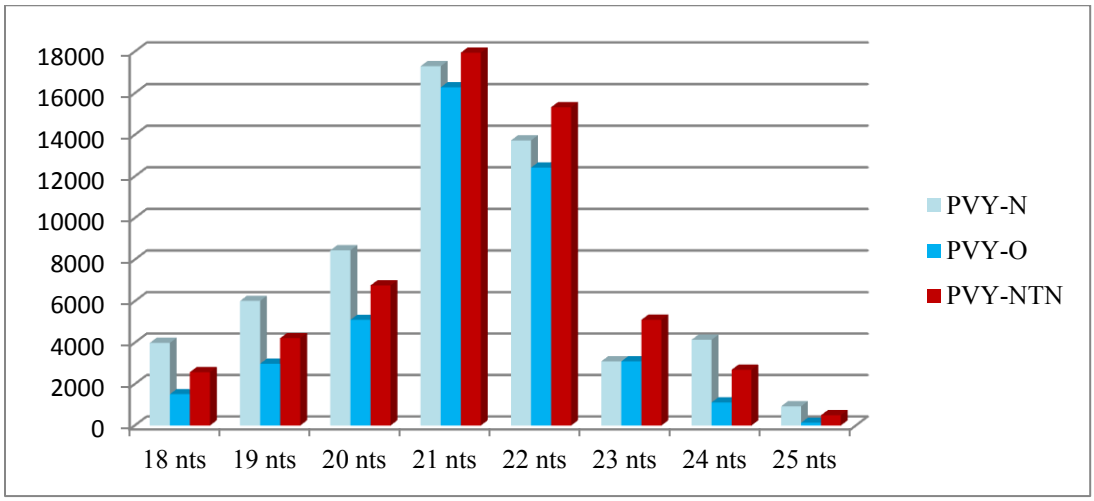

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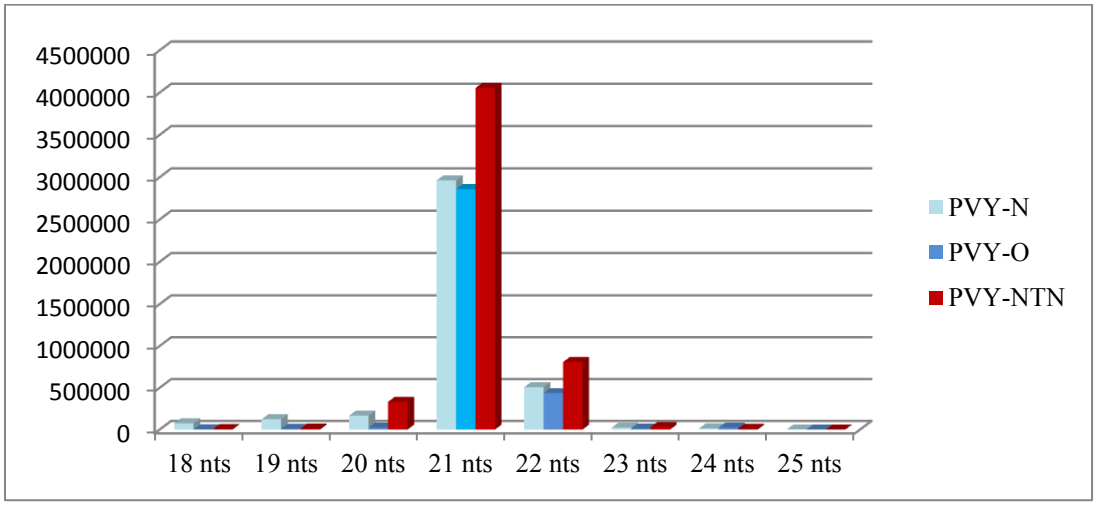

(B)

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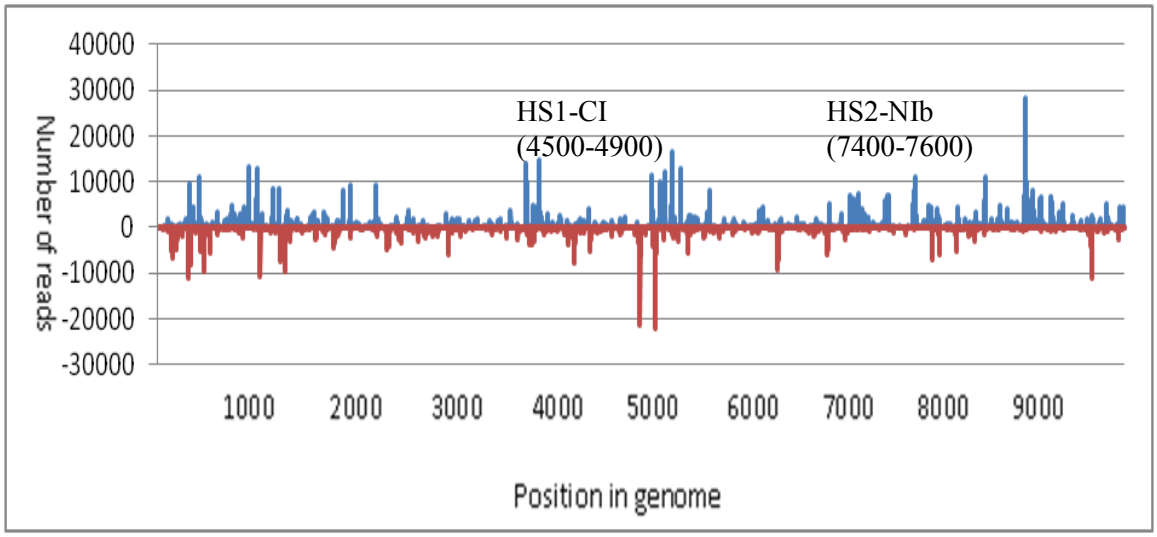
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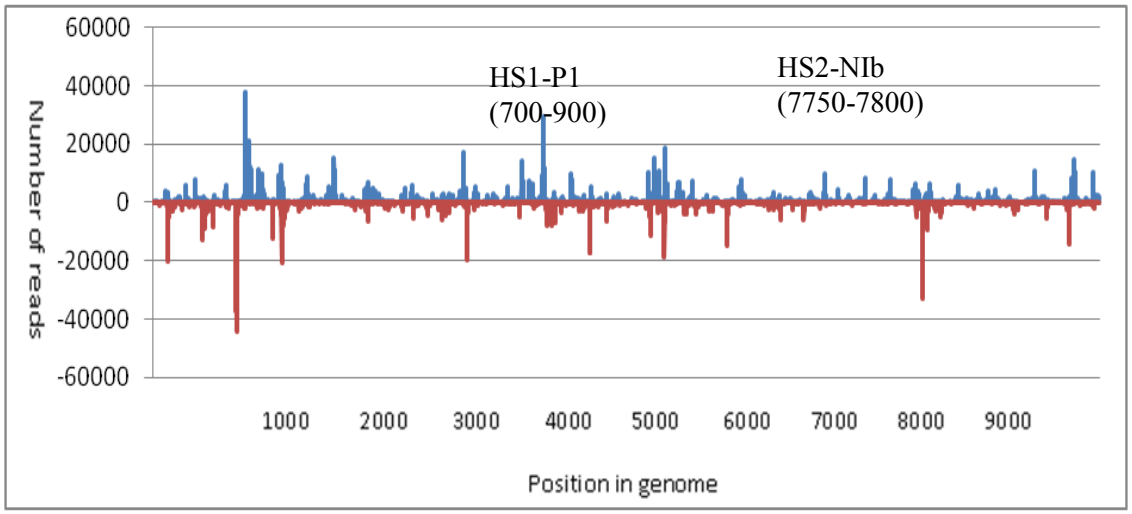
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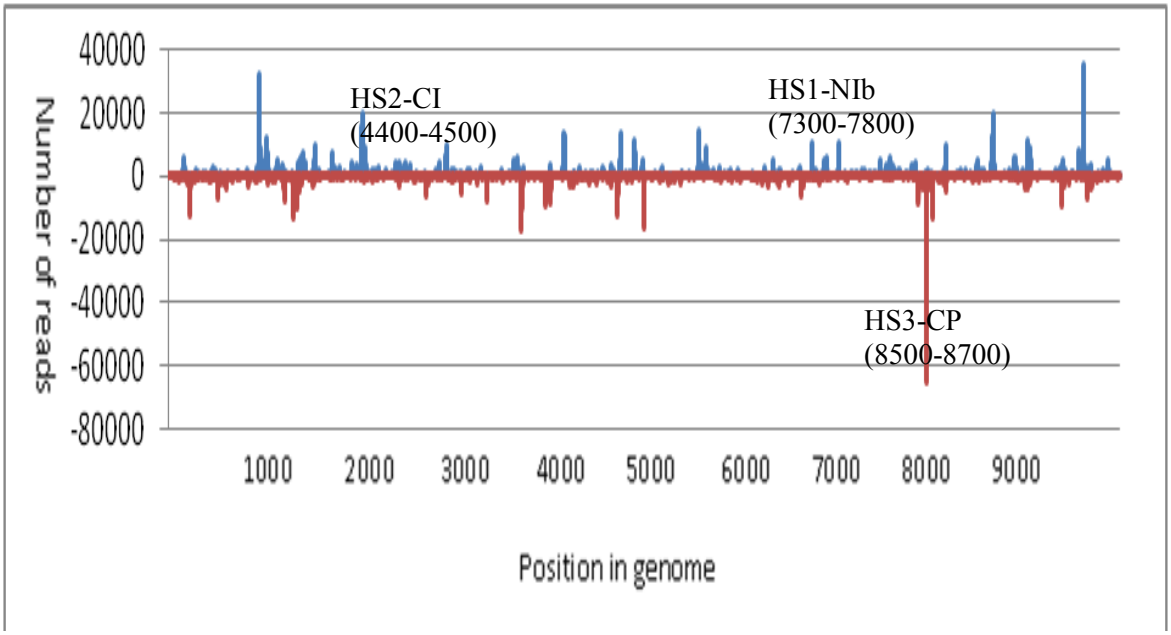

(C)

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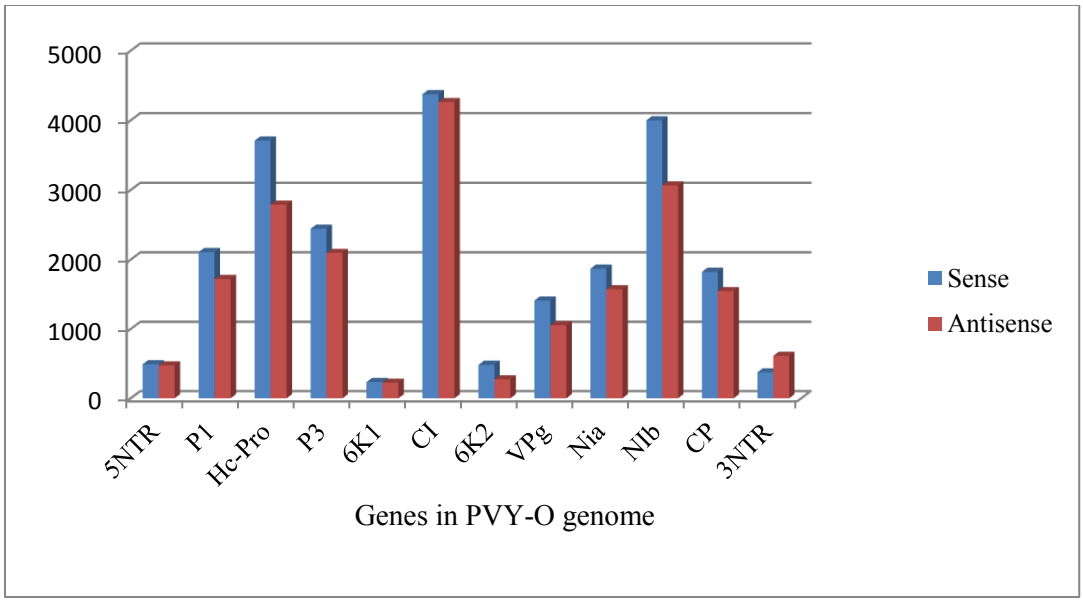
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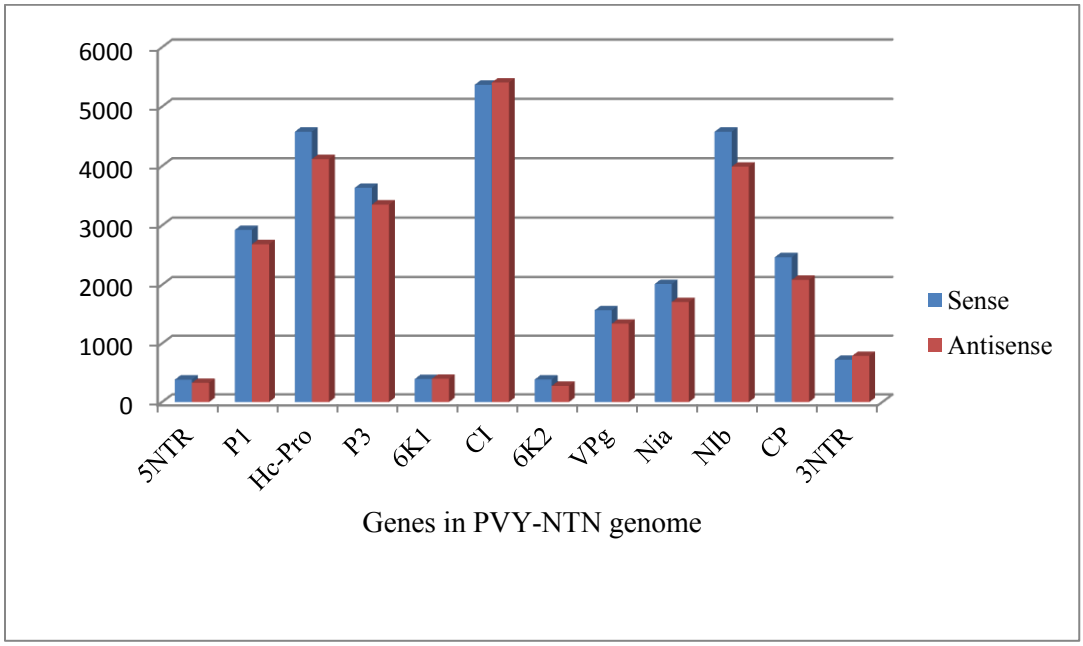
3NTR
Sense
Antisense
Genes in PVY-O genome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Sense
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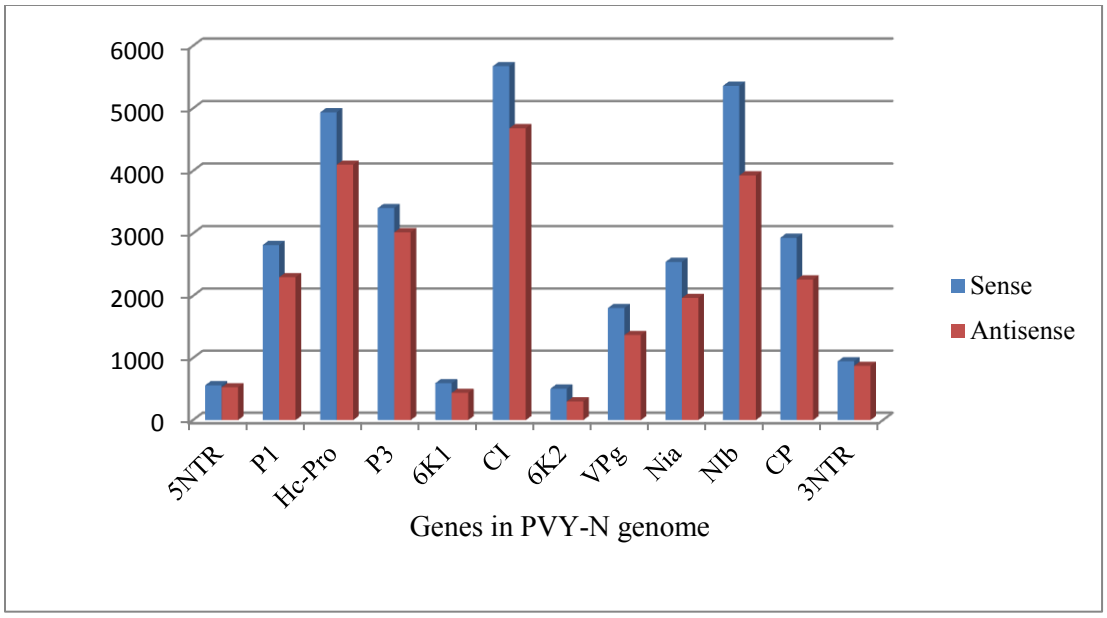
Genes in PVY-NTN genome



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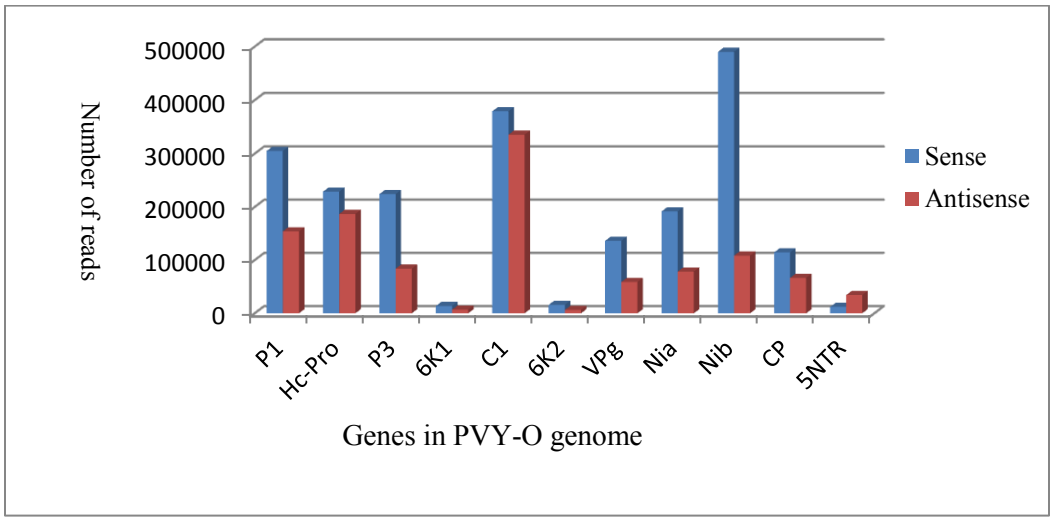
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Number of reads
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P3
6K1
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6K2
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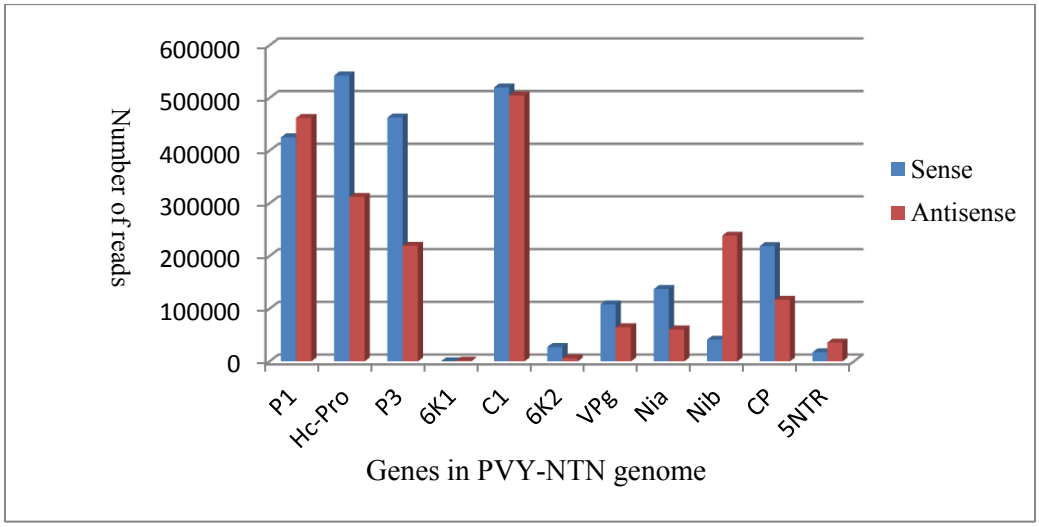
Genes in PVY-O genome
Sense
Antisense

(A)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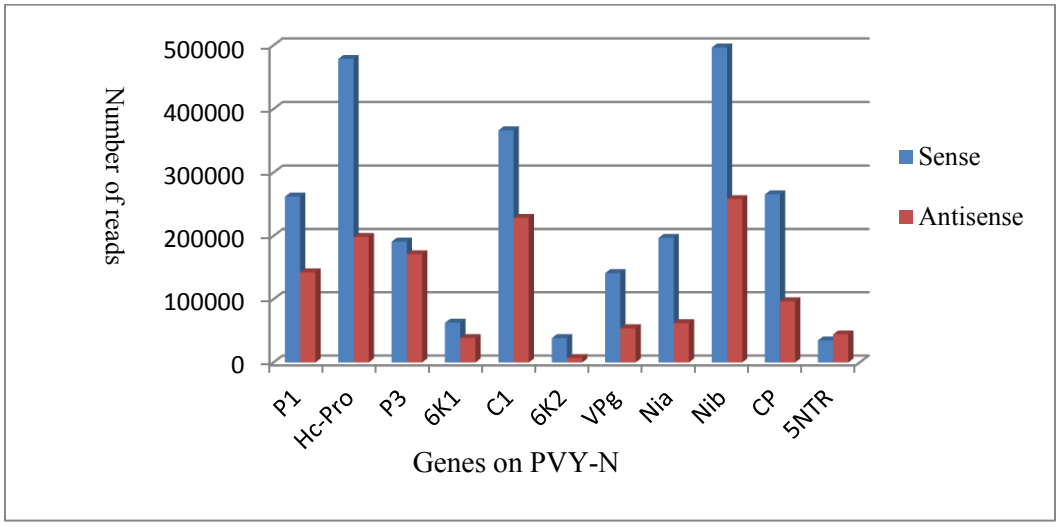
Genes in PVY-NTN genome
Sense
Antisense

(B)



(C)

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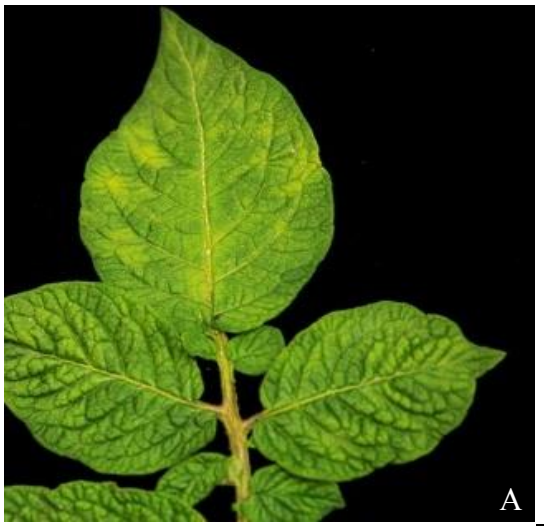

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(http://www.plantwise.org/Uploads/CompendiaImages/Normal/pvy__01.jpg)

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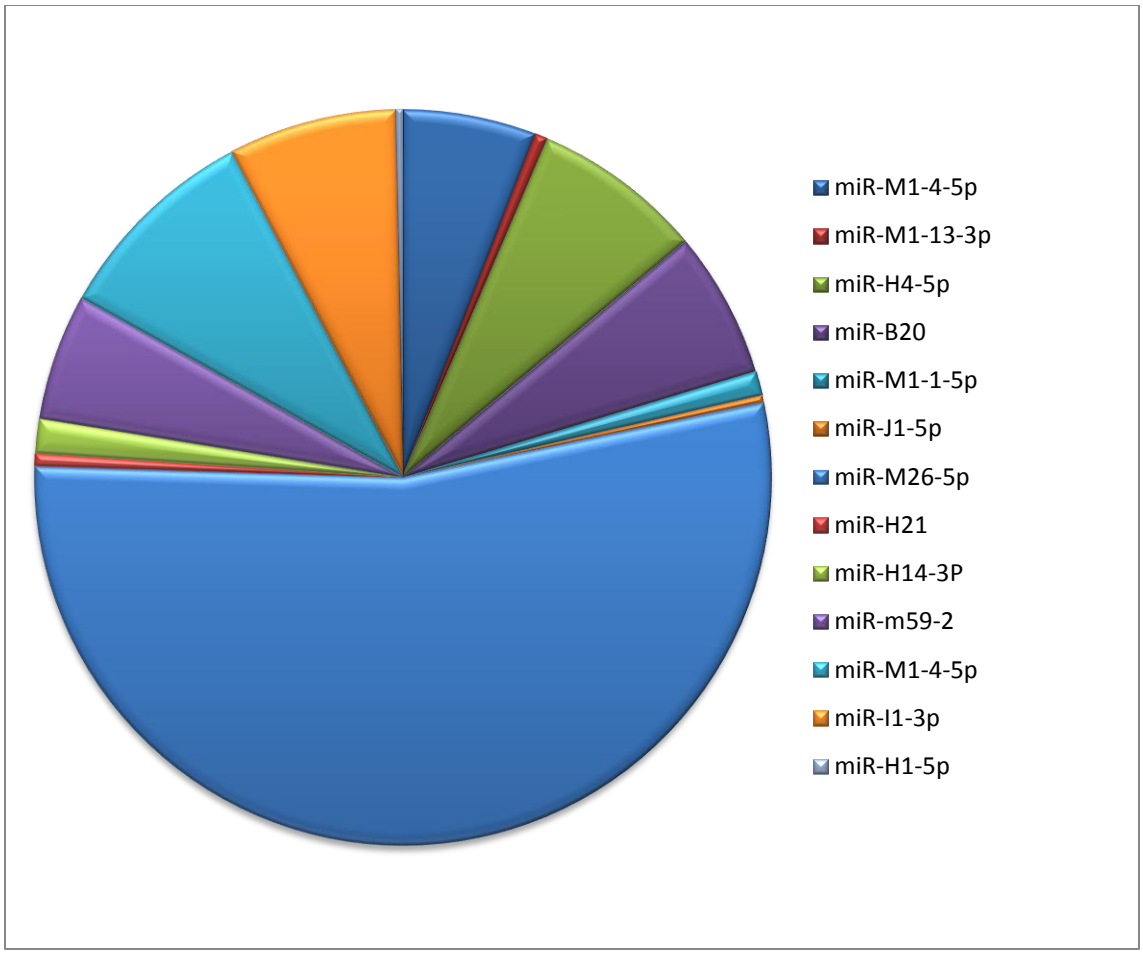


Figure 15: Expression pattern of different microRNAs from 13 different miRNA families in Potato *virus Y* infected and healthy Russet Burbank plants.

CHAPTER THREE

ANTAGONISTIC INTERACTION BETWEEN *POTATO VIRUS S* (PVS) AND *POTATO VIRUS Y* (PVY) IN DIFFERENT GENETIC BACKGROUNDS OF POTATO (*SOLANUM TUBEROSUM* L.)

ABSTRACT

The occurrence of two or more plant viruses in a single plant is a common phenomenon. Multiple virus infections are routine happening in cultivated potato, *Solanum tuberosum*. The interaction between *Potato virus S* (PVS) and *Potato virus Y* (PVY) was investigated in three commercial potato cultivars: Defender, Desiree and Russet Burbank. Mixed infection of PVS and PVY resulted in reduced PVY multiplication in infected plants of Defender, Desiree and Russet Burbank. The symptoms produced by PVY in double infections with PVS were less severe in comparison to the symptoms produced by PVY in single infections. The levels of PVS were similar in single as well as double infections showing that PVY does not have inhibitory effect on the replication of PVS in mixed infections. The symptoms produced by PVS were similar in single as well as mixed PVS and PVY infections. A similar kind of interaction was observed in three cultivars, indicating that the antagonistic effect of PVS on the multiplication of PVY is independent of the genetic backgrounds of potato.

INTRODUCTION

Potato (*Solanum tuberosum*) is a perennial plant from the *Solanaceae* family, that is also called nightshades. It is the world's fourth largest food crop following rice, wheat and maize(UNFAO, The International Year of The Potato, 2008). The family Solanacae includes 2000 species, out of which 100 are tuber bearing. Potato originated in South America, in Peru and Bolivia from where it later spread to other parts of the world. The introduction of potato ouside Andes is probably four centuries old (Neiderhauser, 1993). China is the world's largest potato producer followed by Russia, India and United States and about 1/3rd of world's potato supply comes from India and China (Hijmans, 2001). In 1990's genetically modified potatoes were being used to make frozen products in US. Later, consumer concerns resulted in the decline of GM potatoes for commercial acceptance, however, some procuers cultivate GM potatoes for large scale acreages (Guenthner, 2002). Due to clonal propagation, potato plant is infected by many different pathogens which include bacteria, fungi, nematodes, viruses and viroids (Stevenson *et al*., 2001). China, India, Russia, Ukraine and United States are top five potato producing countries in the world (FAOSTAT data, 2013). Among viruses infecting potato, *Potato virus Y*, *Potato leafroll virus*, *Potato virus X*, *Potato virus S*, *Potato virus M* and *Potato virus M* are economically important viruses. *Potato leafroll virus* is being controlled so economical damage due to PLRV is less due to its control options (Loebenstein and Gaba, 2012)

POTATO VIRUS Y

Potato virus Y (PVY) belongs to genus *Potyvirus* and family *Potyviridae*. The Genus *Potyvirus* is the largest of plant infecting viruses affecting potato crops. There are more than 180 different viruses in this group that cause significant losses agriculture (Ward and Shukla, 1991).

PVY infects economically important plants from the Solanaceae family which include tomato, tobacco, potato and pepper (McDonald and Singh, 1996). A characteristic feature of the viruses from the family *Potyviridae* from other virus groups is the formation of cylindrical inclusions in infected plant cells (Edwardson, 1992)

TRANSIMISSION OF PVY IN POTATO

PVY is transmitted by aphids, through grafting and sap inoculation. Several species of aphids transmit PVY to different hosts in a non-persistent manner. Green peach aphid, *Myzus persicae* is the most efficient vector in transmitting PVY to potato plants. During transmission of PVY, the acquisition and inoculation periods are very short and the aphids remain viruliferous for a very short time (Ragsdale, 2001; Hulbert *et al*., 2003). Aphids can acquire the virus during brief probes of five seconds and longer probes of ten seconds to one minute increase transmission efficiency, however, longer feeding periods from five to ten minutes result in poor transmission. Fasting of aphids prior to virus acquisition increases transmission efficiency (Bradley, 1954). Studies have found that hairy nightshade that is a common weed in the Pcific Northwest can play role in PVY spread as it is favorite host of PVY (Cervantes and Alvarez, 2010).

MOLECULAR BIOLOGY OF PVY

The virus has a single-stranded, flexous rod-shaped, positive-sense RNA genome of about 9.7-kb with a covalently-linked VPg protein at the 5' end and a poly-A tail at the 3'end of the genome (Figure 2). Morphologically, the virions are filamentous which are 680-900 nm in length and 11-15 nm in diameter (Edwardon, 1947: Daughtery and Carrington, 1988). The CIb is considered to be the single most important phenotypic criterion for distinguishing a potyvirus from other virus groups. Single open reading frame encodes a large single polyprotein (~3000

aa) which is processed into 9 functional proteins by three virus-encoded proteases (P1, Hc-Pro and NIb). The cleaved proteins include P1, helper-component protease (Hc-Pro), P3, 6K1, cytoplasmic inclusion (CI) protein, nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb), genome-linked viral protein (VPg) and coat protein (CP). HcPro is involved in aphid transmission of potyviruses by their aphid vectors. These 9 cleaved proteins are involved in different steps of the virus life cycle (Edwardson, 1992; Fauquet *et al*., 2005). The helper component and coat protein is involved in aphid transmission of potyviruses by their aphid vectors. The 5' leader sequence has an internal ribosome entry site (IRES) and cap-independent translation regulatory elements (CIREs). The IRES directs cap independent translation through a mechanism similar to that used by eukaryotes (Ravers *et al*., 1999; Torrance *et al*., 2006).

SYMPTOMS OF PVY IN DIFFERENT HOSTS

Infection with PVY in potato plants results in the development of mosaic, mottling, stunting, leaf malformation, necrotic ring spots, wilting of plants, leaf drop and premature death of plants. Symptoms are dependent on host type, virus isolate and the environment (Delgado and Grogan, 1970). The ordinary PVY-O induces mosaic, mottling and stunting in potato cultivars: Russet Burbank and Red Norland. Most of varieties display no symptoms or show latent infection. These varieties are often referred as PVY carriers which include Gem Russet, Calwhite, GemStar Russet, Russet Norkotah, Shepody and Silverton Russet (Rykbost *et al*., 1999, Nolte *et al*., 2002). In certain varieties like Ranger Russet, PVY infection may cause severe foliar damage, wilting and even death of the entire plant. These symptoms are characteristics of the ordinary strain of PVY referred to as PVY-O, whereas PVY-N or necrotic strains induce veinal necrosis in tobacco. Tubers of infected potato plants do not show visible

symptoms in most instances however, in case of PVY-NTN isolates, the tubers of susceptible potato cultivars produce necrotic rings (Boonham *et al*., 2002).

PVY STRAINS

PVY exists as a complex of strains which are identified on the basis of their reaction in different potato cultivars, tobacco and nucleotide sequences. Three chief strains of *Potato virus Y* have been recognized, Stipple streak strain (PVY-C), ordinary or common strain (PVY-O) and tobacco veinal-necrotic strain (PVY-N). Several recombinant variants PVY-NTN (necrosis tuber-necrotic strain), PVY-N:Wi (necrotic-wingla) and PVY-N:O (recombinat between necrotic and ordinary strain have been identified (Chrzanowska, 1991; Kerlan, 2006; Singh *et al*., 2008). The recombinant strains have emerged recently and have become prevalent in potato fields The necrotic recombiant strain, PVY-NTN has become especially important these days because the tubers of infected plants show necrotic rings which render the potatoes unmarketable thus having a greater economic impact for potato growers (Beczner *et al*., 1984).

A group of isolates which elicit hypersensitive response in potato cultivars with Ny gene like Desiree and Mars Bard was named as PVY-O and isolates which induced HR in potato genotypes with Nc gene like King Edward were named as PVYc (Cockerham, 1943). PVYz was named based on HR reaction produced in potato cultivars with Nz resistant gene in the background (Jones, 1990 and Singh *et al*., 2008). Such isolates which could overcome Ny, Nc, Nz genes and did not elicit HR response in potato genotypes with these resistant genes were further classified based on their symptoms produced in tobacco. The isolates inducing vein necrosis in tobacco were classified as PVY-N (De Bokx, 1961). The genome sequences of PVY-O, PVY-N and PVY-C isolates are known whereas the recombinant isolates were found to have three to four recombinant junctions between parent strains PVY-O and PVY-N. The recombinant

junctions are mostly in the regions of P1, Hc-Pro and VPg-NIa (Hu *et al*., 2009). An isolate of PVY was identified in Idaho that showed different characteristics and it was named as PVY-O (O5). This isolate produces symptoms which resemble symptoms induced by ordinary isolate (PVY-O) but serologically it reacts to PVY-N specific antibodies (Karasev *et al*., 2011). It is important to point out that PVYO-O5 was found ecologically expanding during the 3-year PVY survey (Gray *et al*., 2010), and thus it is hypothesized that PVY-O (O5) has certain evolutionary advantage over the ordinary PVY-O isolates (Karasev *et al*., 2010).

CONTROL OF PVY

Insecticides seem ineffective in controlling PVY because the time required to acquire the virus and transmit it is within seconds to minutes. Application of insecticide (Karate Zoen), elicitor (Bion) and mineral oil (Telmion) in controlling PVY in potato fields showed that elicitor treatment was ineffective in controlling PVY and similarly insecticide treatment did not give adequate control of aphid infestation. Mineral oil application reduced PVY spread but was ineffective in controlling aphid populations. Treatment consisting of insecticides gave inadequate protection from aphid infestations (Dupis *et al*., 2013). Another study reported that insecticides can provide some control against PVY spread in potato fields from infected to healthy plants if there is no external source of PVY (Milovesic *et al*., 2012).

For managing PVY, it is important to use disease free seed potatoes with zero or extremely low virus titers. Spread of PVY through aphids is from preexisting infected plants in the same field as they don't carry the virus from outside sources. Removal of infected plants is an effective way for controlling PVY especially early in the season and it will slow down PVY spread in the field (Nolte *et al*., 2009). Infected seed tubers are the most important source of PVY. Other plant species including hairy nightshade also play an important role in PVY spread.

Hairy nightshade is an important weed in the Pacific Northwest and serves as an important virus reservoir (Cervantes and Alvarez., 2010). Crop borders which means a non-PVY host crop between the early planted seed lot and the aphid flights provides a buffer. Aphids usually land between the fallow land and the green crop. Landing of aphids in crop borders helps in cleaning their stylets thus resulting in less PVY spread (Difonzo *et al*., 1996; Boiteau *et al*., 2009).

EPIDEMIOLOGY OF PVY

Hairy nightshade (*Solanum sarrachoides*) plays role in the epidemiology of *Potato virus Y* by providing a niche to aphids: Green peach aphid and potato aphid (*Macrosiphum euphorbiae).* It was found that PVY-NTN multiplied at higher rates in hairy nightshade in comparison to potato plants. Further, the transmission efficiency of green peach aphid was increased when it fed on PVY-infected hairy nightshade (Cervantes and Alvarez., 2010). Raccah *et al.,* (1985) studied transmission efficiency of forty four aphid species during three year period in pepper fields. Nineteen aphid species transmitted *Cucumber mosaic virus* (CMV) whereas seven species transmitted *Potato virus Y* (PVY). Maximum infection of CMV and PVY was caused by *Aphis citricola, Myzus persicae* and *Macrosiphum euphorbiae. A. citricola* was the most common aphid species in flight whereas *Myzus persicae* was present at high rates in pepper plants. *A. citricola* and *Aphis* spp. transmitted more than 50% of CMV in 1981 and 1982 and of PVY in 1981. Crop borders of soybean and wheat can reduce PVY incidence in potato fields. A study conducted over two year period found that aphid landing rates in fallow bordered or crop bordered potato fields were not significantly different, however, the incidence of PVY in potato crops was considerably less. The study showed that crop borders of soybean or wheat can be used as a way to reduce PVY incidence (Difonzo *et al*., 1996).

POTATO VIRUS S

PVS (Genus *Carlavirus*, Family *Betaflexiviridae*) is another important virus distributed worldwide infecting potatoes. PVS was first time recognized in in 1952 (Brunt and Loebenstein, 2001; Jones, 1981). In temperate regions, the only host is potato, although there are other hosts in tropical climates. PVS alone can cause 3-20% tuber yield loss (Wetter, 1971). PVS can infect plants from the Chenopodiaceae and Solanaceae families. Different strains of the virus are recognized including the ordinary (PVS-O) and Andean (PVS-A) strain groups. PVS-O induces local lesions on inoculated leaves of *Chenopodium* spp whereas the Andean strain (PVS-A) induces systemic chlorosis in *Chenopodium* (De Bokx, 1970). The two strains can be identified on the basis of the differences in nucleotide sequences of the 7K protein, CP and nucleotide-binding (11K) protein at the genetic level through nucleic acid spot hybridization (Foster and Mills, 1992)

TRANSMISSION OF PVS

The virus is transmitted through mechanical contact, grafting, through infeted seed tubers and by aphids in a non-persistent manner, mainly by *Myzus persicae* (Stevenson *et al*., 2001, Fletcher, 1996). *Myzus persicae* and some other aphid species have been found transmitting PVS to potato plants. Andean strain spreads faster than ordinary strain due to higher levels in infected potato plants (Wardrobe *et al*., 1992).

GENOME OF PVS

PVS genome is single-stranded, positive sense, RNA molecule that is about 8.5 Kb in length. Genome has 6 open reading frames (ORFs), 5'-cap and 3'-end poly-A tail (Figure 1). The genomic RNA is encapsidated in 33K protein and it has open reading frames (ORFs) which

encode polypeptides with Mr. of 10734, 32515, 7222, 11802, 25092 and more than 41052. The latter ORF encodes amino acid sequences similar to those of putative viral replicase genes and the Mr. 32515 polypeptide is found to be the virus coat protein. Recent nucleotide sequence data from PVS and a related carlavirus, *Potato virus M* (PVM) (Rupasov *et al*., 1989), demonstrated that these two viruses have genome organizations similar to that of *Potato virus X* (PVX) (Mackenzie *et al*., 1989; Huisman *et al*., 1988) and other members of the *Potexvirus* group. It was found that a major amino acid block present at the 3'end of genome differs between ordinary (PVS-O) and Andean strain (PVS-A). The difference in symptomology and aphid transmission might be due to these amino acid differences (Foster and Mills, 1992) .

SYMPTOMS

The ordinary strain is symptomless in most potato cultivars whereas PVS-A produces stronger symptoms in infected potato plants. PVS-A differs from PVS-O in producing stronger symptoms in secondary infected potato plants and by reaching higher concentrations in the leaves. PVS-O is symptomless in majority of potato cultivars, with occasional symptoms of vein deepening, rugosity and leaf bronzing. The symptoms of PVS-A include premature senescence, loss of leaves especially in secondary infected plants. Symptoms development is highly dependent on environment (Dolby and Jones, 1987; Rose, 1983, Jeffries, 1998).

MANAGEMENT OF PVS

Spread of PVS in fields should be minimized through mechanical contact and through injuries. Insecticides are not effective in controlling PVS but crop oils can be used to minimize its spread. Removal of visibly diseased plants from the field as soon as possible is recommended.

Removal of volunteer potatoes from the field is also useful for the control of potato viruses (Burrows and Zitter, 2005). Mixed infection by plant viruses is a common phenomenon and a number of plant diseases are a result of multiple pathogen infections (DaPalma *et al*., 2010; Waner, 1994). In case of mixed virus infections, multiple outcomes are found which include synergism, in which one virus or both viruses facilitate each other's replication or transmission into the host plant, a phenomenon known as helper dependence and antagonism, one virus is mainly at the advantage whereas the replication of the other virus is reduced or the virus is unable to enter the host plant, a phenomenon known as mutual exclusion (Garcia-Cano *et al*., 2006; Ranteria-Cannet *et al*., 2011; Untiverous *et al*., 2007). Cross-protection, a form of antagonism, is also observed in which an early infecting virus strain which is in fact less severe protects the plant from later infecting severe strain of the same virus. This phenomenon is usually found between the strains of the same virus. This technique has been used as way to protect field crops or vegetables from the severe strains of viruses in which plants are inoculated with mild strains of the virus (Fulton, 1986, Hanssen *et al*., 2010; Lecoq and Rakkah, 2001). In cross-protection, one virus protects the host from infection with more severe virus resulting in reduced disease symptoms and damage. Both of the viruses replicate in the host and move from cell to cell, similar to vaccination concept in humans (Ziebell and Carr, 2010). In field conditions, infected citrus plants with mild strains of *Citrus tristeza virus* (CTV) protect the plants against the severe strains of CTV. The phenomenon of cross-protection among CTV isolates was observed only between the isolates of the same strain and not between isolates of different strains (Folimonova *et al*., 2010). Reombination is also one of the outocmes of mixed vral infections as result of which novel strains of viruses have emerged. Mixed virus infections produce recombinant strains which are more virulent than parental strains for their fitness. This

phenomenon has become very widespread due to monoculture of crops and lack of genetic diversity in cultivated crops (Berrie *et al*., 2001; Hou and Gilbertson, 1996). Recombiannt strains have led to breakdown of resistance in commercial varieties of several impartant crops like in Potato to *Potato virus Y* (PVY), in cotton to *Cotton leaf curl virus* (CLCV) and in cassava to *Cassava streak virus* (CaSV). The mixed infections result in increased disease severity resulting in huge yield losses (Mendez-Lozano *et al*., 2003; Pita *et al*., 2001).

Another example of synergism is found in Potato viruses: *Potato leaf roll virus* (PLRV) and *Potato virus Y* (PVY). Plants with mixed infections of PVY and PLRV developed more severe symptoms than single infections of each virus. However, no differences were observed in virus titers in single and mixed infections. Mixed infections of PVY and PLRV resulted in increased fecundity of green peach aphid and potato aphid was increased. Aphid vectors setlled more on plants doubly infected with PVY and PLRV(Srinivasan and Alvarez, 2007). Mixed geminivirus infections of *East African cassava mosaic cameron virus* and *African cassava mosaic virus* in cassava and tobacco, resulted in increased accumulation of both viruses as well as stronger symptoms. It was found that in mixed infections, virus encoded prodcuts caused a suppression of host post-transcriptional gene silencing mechanism (Vanithrani *et al*., 2004). Suppression of host defense mechanism is supported by another study in which synergism was found between *Tomato spotted wilt virus* (TSWV) and *Iris yellow spot virus* (IYSV). TSWV causes systemic infection in *Datura* wheras IYSV produces local infection in the same host. Mixed infection of both viruses resulted in systemic spread of IYSV in Datura. Analysis showed that there was reduced accumulation of small interfering RNAs (siRNAs) from the NSs silencing suppressor which resulted in suppression of the host defense mechanism (Pappu *et al*., 2012). Antagonism is also observed in case of mixed virus infections in plants. Barley plants with

mixed infection of three different strains of *Barley yellow dwarf virus* (BYDV) showed less disease symptoms in comparison to single infections of each strain (Jedlinski and Brown, 1965). Likewise, when *N. benthamiana* plants were doubly inoculated with cDNA clones of potyviruses: *Plum pox virus* (PPV), *Tobacco vein mottling virus* (TVMV) and *CIover yellow vien virus* (ClYVV) expressing green and red fluorescent proteins (GFP and RFP) or with identical but differently labelled potyviruses (e.g. PPV-GFP and PPV-RFP), the two viral populations competed with each other during the colonization of epidermal cells (Dietrich and Maiss, 2003).

POTATO CULTIVARS USED IN THE STUDY

Defender was developed in 1994 by J.J. Pavek as a result of hybridization between clone KSA195-90 and Ranger Russet. It is the only potato cultivar with foliar and tuber resistance to late blight. The name Defender refers to its foliar and tuber resistance to late blight fungus *Phytophthora infestans*. It is high yielding variety which gives higher yields than Russet Burbank, Ranger Russet and Shepody and is mainly used for french fries and other frozen potato products. Defender has white skinned potato tubers which are long (Novey *et al*., 2006).

Desiree is a main crop potato variety originally bred in Netherlands in 1962. It has yellow flesh with a distinctive flavor and is resistant to drought. It has medium height and is spreading in nature. The flowers are red/purple on hairy stalks. The tubers have red smooth skin, round to oblong tubers. It is an early maturing variety that takes 80-100 days to reach maturity and is resistant to many common potato diseases. This is a versatile potato variety with all cooking purposes including baking and roasting. The plants have resistance to drought. It shows hypersensitive response to ordinary strain of *Potato virus Y* (The European Cultivated Potato Database, 2012).

Russet Burbank is the main cultivar grown in Idaho, Washington, Colorado and New Brunswick Canada. Russet Burbank accounts for nearly 40% potato production in United States. It was developed in 1914 by Luther Burbank. Plants produce large tubers with russeted skin, white flesh and with numerous eyes. It is a late maturing potato variety with indeterminate growth habit that takes 140-150 days to reach maturity and has resistance to potato scab and net necrosis. The main uses include french fries and for baking. The starch and sugar content in tubers are medium compared to other potato varieties. Russet Burbank is also known as "Idaho potato (USDA-ARS National Genetics Resource Program GRIN, 2013; The Potato Association of America, 2009).

In this study, I hypothesized that *Potato virus S* and *Potato virus Y* and PVY might interact with each and with host at physiological and molecular levels resulting in different disease phenotypes. For this purpose, I used three different potato cultivars to examine the interaction in different genetic background of potato.

MATERIALS AND METHODS

COLLECTION AND MAINTENANCE OF VIRUS ISOLATES

Potato virus Y (PVY) isolate from the ordinary strain (PVY-O) was obtained from Dr. Alexander Karasev's lab from University of Idaho and was maintained in *Nicotiana tabacum* in growth chamber. *Potato virus S* (PVS) isolate was obtained from PVS infected potato tubers obtained from potato fields in Idaho and was maintained in *Chenopodium quinoa* in greenhouse under natural conditions.

PLANT MATERIALS AND VIRUS INOCULATIONS

Three potato cultivars Russet Burbank, Defender and Desiree were grown in greenhouse under natural day light conditions in an insect free area. At 6 weeks-stage, the plants were inoculated with virus inocula and were kept in the greenhouse. The virus inocula were prepared by homogenizing 1 gram of leaf tissue infected with each virus PVS and PVY in 0.1M phosphate buffer (1:5 {wt/vol} tissue: buffer) with 0.4% beta mercaptoethanol added. Before inoculations with *Potato virus S* and *Potato virus Y*, plants were tested by ELISA to make sure that the plants were free of infection from any *Potyvirus* or *Carlavirus.* For each treatment, 6 plants were maintained and four leaves of each plant (Figure 3) were inoculated by dusting with celite 545 and carborundum 320 grit (Fisher, Scientific, Fair Lawn, NJ) mixture (1:1{ wt/wt}). Likewise, in each treatment, uninoculated healthy controls were also included. Three different treatments were maintained. In first and second treatments, plants were inoculated with PVS and PVY only whereas in the third treatment, plants were co-inoculated with PVS and PVY (Tables 1, 2 and 3). The experiment was repeated three times at different times of the year.

SYMPTOMS OBSERVATION AND ENZYME-LINKED IMMUNOSORBANT ASSAY

After inoculations with respective viruses, symptom observation commenced two weeks post-inoculation. The plants started showing symptoms of infection two weeks-post inoculation which included mosaic, mottling, stunting and bronzing symptoms in case of single as well as mixed PVY and PVS infections. At three weeks post-inoculation, two systemic leaves (Figure 3) were taken from each plant to test by ELISA by using Agdia kit (Elkhart, IN) and the ELISA values were recorded at 405 nm. Positive and negative controls were included in each ELISA assay and a sample was considered as positive if its ELISA value exceeded three times the OD

value of the healthy sample. For ELISA testing, it was made sure that the same amount of plant tissue was ground in extraction buffer. For this purpose, the infetced tissue was weighed that was 0.50 gram for infected samples and for the healthy samples the same amount of tissue was ground in extraction buffer. In healthy samples, leaves from uninfected potato plants were used. The ELISA plate was read under the ELISA reader at 405 nm light wavelength and intensity of emitted light was measured. The experiment was conducted three times at different times of the year as explained in the figure legends (Figure 3-6). The cross-reactivity of PVY and PVS antisera was also checked and the results are shown (Figure 7).

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was used to test the significance of the data and the results showed that the means were significantly different at 0.05 level of probability from healthy and negative controls (Neter J, 1990). The experiment was repeated three times and each experimental repeat has data from six replicates (plants). The data was represented in graphical form for each treatment and variety by taking average values of each treatment (Figures 3-6). The standard deviation of each treatment was also calculated.

RESULTS

SYMPTOM OBSERVATION AND DISEASE DEVELOPMENT

Three weeks post-inoculation with PVS and PVY, plants were observed daily for symptoms development and infections symptoms were recorded. Symptoms of infection started to appear two-weeks post inoculation and became severe with the passage of time. In case of PVY singly infected plants, symptoms included mosaic, mottling, stunting and wilting of entire

plants (Figure 2B, C and D). Some of the plants died with the increasing number of days post-inoculation.The plants of Desiree showed necrotic lesions 20 days post-inoculation which is a hypersenstive response to PVY-O infection. Most of Deisree plants showed severe stunting and wrinkilng of leaves three weeks post-inoculation as a result of infection with PVY. In plants infecetd with PVS only, most of the potato plants did not show any symptoms of infection. PVS was symptomless in Russet Burbank and Desiree. The cultivar Defender showed systemic necrosis and leaf bronzing spots as a result of infection with PVS (Figure 2E, F and G).

In case of mixed infection with PVS and PVY, mosaic and mottling which are common symptoms as a result of infection with PVY-O strain, the symptoms were mild in comparison to plants infected with PVY alone. PVY symptoms were most visible in Russet Burbank which showed systemic mottling, mosaic and plant stunting as a result of PVY-O infection. The symptoms of PVY infection were not visible in case of Defender which did not show any symptoms of the infection. The symptoms of PVY were severe in single infections versus mixed infections with PVS symptoms were not altered in sinlge as well as dual infections with PVY.

ENZYME-LINKED IMMUNOSORBANT ASSAY TO DETERMINE RELATIVE VIRUS LEVELS

Three weeks-post inoculation, one to two leaves from each plant were taken to test for infection with inoculated viruses respectively. The samples were tested in dupliacte wells using commercially available kit (Agdia, Elkhart, IN) following exactly the kit protocols. Healthy uninfecetd potato samples were included as healthy controls and only buffer was included as negative control. The ELISA results showed that plants doubly infected with PVS and PVY had lower levels of PVY in comparison to plants infected with PVY alone. The ELISA values for PVY infected plants ranged from 3.80 to 4.00. The OD values for PVY antiserum from PVS plus

PVY doubly infected plants were 1.50 to 2.00 which were much lower than OD values in PVY single infections. The OD values for PVS and PVY doubly infected potato plants were similar among three different cultivars and this showed that the interaction between PVS and PVY is similar in three different potato cultivars and is not host-dependent. The OD values for PVS infected samples were in the range of 1.20-1.80 in case of plants infected with PVS only. The relative levels of PVS were very similar in case of sinlge or double infections with PVY among three different cultivars. This showed that PVS is not being affected with regard to its replication in Defender, Desiree and Russet Burbank potato plants by PVY. The lower levels of PVY in case of double infections with PVS are in accordance with PVY mild symptoms produced in infected plants and this showed that PVS has an antagonistic effect on the multiplication of PVY. In dual infections.The antagonistic effect is not affecetd by different backgrounds of potato backgrounds.

DISCUSSION

The interaction between *Potato virus S* (PVS) and *Potato virus Y* (PVY) in three commercially grown potato cultivars: Defender, Desiree and Russet Burbank was studied. In case of PVY singly infecetd plants, symptoms were more severe which included mosaic and mottling of leaves, stunting and wilting of infected plants whereas some of the plants died with the increasing number of days post-inoculation. PVS did not produce symptoms in Russet Burbank and Desiree whereas Defender developed leaf bronzing spots. More severe symptoms in PVY single infections showed that PVS has antagonistic effect on the replication in mixed infections. The ELISA results showed that plants doubly infected with PVS and PVY had lower

titers of PVY in comparison to plants infected with PVY alone. ELISA values for PVY infected plants ranged from 0.380 to 0.40. The absorbance values for PVY antiserum from PVS plus PVY doubly infected plants were 0.150 to 0.200. The OD values for PVS and PVY were similar among three varieties and this showed that the interaction between PVS and PVY is similar in three cultivars and is not cultivar dependent. The ELISA OD values for PVS infected samples were less and they were in the range of 0.12-0.18 in case of plants infected with PVS only. Titers of PVS were similar in case of sinlge or double infections with PVY in three cultivars and this showed that PVS replication is not being affected in Defender, Desiree and Russet Burbank plants. Low levels of PVY in doubly infected plants suggested that PVS is acting against PVY multiplication in doubly infected plants and has some kind of antagonistic effect on PVY. Whereas PVS levels were simialr in doubly infected plants and singly infected plants with PVS (Figures 4-6). There were no significant differences in virus titers with respect to three different varieties (Figures 4-6). These findings show that in mixed infections, PVS acts antagonistically towards PVY multiplication.

Our findings contrasted with the fidings of Gonzalez-Jara *et al*., (2004) in which they found that the interaction between *Potato virus X* and *Potato virus Y* was host-dependent. There was an enhancement of disease symptoms in *N. tabacum* plants infected with PVX and PVY. Synergistic interaction between PVX and PVY resulted in 10-fold increase in the titre of PVX compared with single infections. In contrast, no marked increase in the titers of PVX was recorded in *N. benthamiana* plants with mixed infections of PVY, *Tobacco etch virus* (TEV) or *Plum pox virus* (PPV). It was concluded that interaction between PVX and a potyvirus is host dependent (Gonzalez-Jara *et al*., 2004). Interaction of *Tomato chlorosis virus* (ToCV) and *Tomato infectious chlorosis virus* (TICV) is also host-dependent. In doubly infected *N.*

benthamiana plants, TICV titers increased and ToCV titers decreased, when compared with concentrations in singly infected plants. In co-infected *Physalis wrightii* plants, titers of both viruses decreased. The pattern of TICV-ToCV-host interactions suggests the existence of differences between the two viruses in adaptation to different hosts (Wintermantel *et al*., 2008). Host-dependent alteration of symptoms has been reported for the plants co-infecetd with *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV), because synergism was observed in *N. tabacum* and *N. benthamiana*, whereas antagonism was found in pepper (Mendez-Lozano *et al*., 2003). A synergy pattern is not only dependent on the host species, but also on the host cultivar, as recently reported for three wheat cultivars co-infected with *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV). The synergistic interaction between both viruses: WSMV and TriMV was found to be cultivar dependent (Tatineni *et al*., 2010).

In our study, mixed infection of PVS and PVY resulted in reduced PVY multiplication as well as less severe PVY symptoms. This highlights the importance that symptomatology is not a reliable criterion to detect PVS or PVY infected potato seed lots. In most cases, the interaction is synergistic between interacting viruses in a single plant, but our findings indicate antagonistic interaction between PVS and PVY. The antagonistic effect of PVS on PVY can provide some valuable clues for controlling PVY epidemics (Garcia-Arenal *et al*., 2003). The mechanism of antagonistic effect needs to be studied which will help to control both potato viruses; PVS and PVY through better management options. Mixed infections of PVS and PVY resulted in less severe PVY symptoms and this can mask the symptoms of PVY in potato cultivars which show PVY symptoms and are used to identify diseased plants. Interaction among viruses is crucial for the understanding of viral pathogenesis and evolution and consequently for the development of efficient and stable control strategies (Read and Taylor, 2001; Renteria- Canett *et al*., 2011).

The biological and epidemiological consequences of antagonistic virus interactions are unforeseeable. Till now, the mechanisms for antagonistic interactions between plant viruses in mixed infections are partly recognized and thus require further detailed studies. Our study provides information about the interaction of PVS and PVY in co-infections in potato crop. Information about the interacting viruses in mixed infections is important to accurately diagnose infected plants having multiple virus infections.

REFERENCES

Adams MJ, Antoniw JF, Bar-Josepha M, Brunt AA, Candresse T, Foster GD, Martelli GP, Milne RG, Milne GR and Fauqet CM. (2004). The new plant virus family *Flexiviridae* and assessment of molecular criteria for species demarcation. Archives of Virology.149: 1045-1060.

Agrios GN. (2005). Plant Pathology (5th edition). Elsevier-Academic Press, San Diego, CA. 922pp.

Bag S, Mitter N, Eid S, and Pappu HR. (2012). Genetic complementation between two tospoviruses facilitates the systemic movement of a plant virus silencing suppressor in an otherwise restrictive host. PLOS ONE: 7(10): e44803

Bradley RHE. (1954). Studies on the mechanism of transmission of *Potato virus Y* by the green peach aphid, *Myzus persicae* (Sulz.) (Homoptera: Aphididae). Canadian Journal of Entomology. 32: 64-73.

Chakraborty S, Vanitharani R, Chattopadhyay B and Fauquet CM. (2008). Supervirulent pseudorecombination and asymmetric synergism between genomic components of two distinct species of begomovirus associated with severe Tomato leaf curl disease in India. Journal of General Virology. 89: 818-828.

Cervantes FA and Alvarez JM. (2010). Role of hairy nightshade in the transmission of different *Potato virus Y* strains on *Solanum tuberosum* (L.) Plant Health Progress. doi:10.1094/PHP-2010-0526-05-RS.

Cockerham G (1943). Potato breeding for virus resistance. Annals of Applied Biology. 30: 105-108.

Delgado-Sanchez S. and Grogan RG. (1970). *Potato virus Y*. CMI/AAB Descriptions of plant viruses. 37: CMI/AAB, Kew, Surrey, England, 4 pp.

Dietrich C. and Maiss E. (2003). Fluorescent labeling reveals spatial separation of potyvirus populations in mixed infected *Nicotiana benthamiana* plants. Journal of General Virology. 84: 2871-2876.

Difonzo CD, Ragsdale DW, Redcliffe ED, Gudmestad NG and Secur GA. (1996). Crop borders reduce PVY incidence in seed potato. Annals of Applied Biology. 129 (2): 289-302.

Dolby CA and Jones RAC. (1987). Occurrence of the Andean strain of *Potato virus S* in imported potato material and its effects on potato cultivars. Plant Pathology. 36: 381-388.

Edwardson JR (1992). Inclusion bodies. Archives of Virology Supplementum. 5: 25-30

Fauquet CM, Mayo MA, Manil off J, Dessel BU and Ball LA. (2005). Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. Amsterdam, the Netherlands: Elsevier Academic Press.

FAOSTAT: Production-Crops, (2010). Food and Agriculture Organization of the United Nations. 2011.

Foster GD. (1992). Molecular variation between ordinary and Andean strains of Potato virus S. Research in Virology. 142(5): 413-416.

Garcia-Arenal F, Fraile A, and Malpica JM. (2003). Variation and evolution of plant virus populations. Int. Microbiology. 6: 225-232.

Garcia-Cano E, Resende RO, Fernandez-Munoz R, and Moriones E (2006). Synergistic interaction between *Tomato chlorosis virus* and Tomato spotted wilt virus results in the

breakdown of resistance in tomato. Phytopathology 96: 1263-1269.

Gonzalez-Jara P, Atencio FA, Martinez-Garcia B, Barajas D, Tenllado, F, and Diaz-Ruiz JR. (2005). A single nucleotide mutation in the *Plum pox virus* HC-Pro gene abolishes both synergistic and silencing suppression activities. Phytopathology. 95: 894-901.

Gray SM, DeBoer SH, Lorenzen J, Karasev AV, Whitworth J, Nolte P, Singh RP, Boucher A, Xu H. (2010). *Potato virus Y*: A significant and evolving threat to potato crops in the United States and Canada. Plant Disease. 94:1384–1397.

Guenthner JF. (2002). Consumer acceptance of genetically modified potatoes. American Journal of Potato research. 79: 309-316.

Hawkes, JG. (1992). History of the Potato. In 'The potato crop'ed. P. Harris. Champan and Hall, London.

Hinostroza AM. (1973). Some properties of *Potato virus S* isolated from Peruvian potato varieties. Potato Research 16: 244-250.

Hua X, Meachama T, Ewinga L, Gray SB, and Karasev A. (2009). A novel recombinant strain of *Potato virus Y* suggests a new viral genetic determinant of vein necrosis in tobacco. Virus Research 143: 68–76.

Hulbert SE, Corsini DL and Wiebe MA (2003). *Potato virus Y* transmission efficiency for some common aphids in Idaho. American Journal of Potato Research. 80: 87-91.

Jeff W, Sri H, and Tom G. (1997). Winged aphids and *Potato virus Y* transmission in Wisconsin. Department of Entomology and Plant Pathology, University of Wisconsin, Madison.

Jones RAC. (1987). Problems associated with *Potyviruses* in potato certification - field inspection and serological testing. EPPO Bulletin 17: 61-67.

Karasev AV, Nikolaeva OV, Hu X, Sielaff Z, Whitworth J, Lorenzen JH, Gray SM. (2010). Serological properties of ordinary and necrotic isolates of Potato virus Y: A case study of PVYN misidentification. American Journal of Potato Research. 87:1–9.

Kerlan C. (2006). Description of Plant Viruses: *Potato virus Y*. Association of Applied Biologists. [http://www.dpvweb.net/dpv/showdpv.php?dpvno=414].

Kohli Pawanexh. (2009). Potato Storage and Value Preservation: The Basics. Crosstree Techno-visors.

Milosevic D, Stamenkovic S, and Peric P. (2012). Potential use of insecticides and mineral oils for the control of transmission of major aphid-transmitted potato viruses. Pesticide and Phytomedicine 27(2): 97-106.

Mendez-lozano J, Torres-Pacheo I, Fauquet CM. and Rivera-Bustamante RF. (2003). Interactions between geminiviruses in a naturally occurring mixture: *Pepper huasteco virus* and *Pepper golden mosaic virus*. Phytopathology, 93 (3): 270-277.

Manzer FE, Merriam DC, and Helper DR. (1978). Effects of *Potato virus S* and the two strains of *Potato virus X* on yields of Russet Burbank, Kennebac, and Kathadian cultivars in Maine. American Potato Journal. 55(11): 601-609.

McDonald JG and Singh RP (1996). Host range, symptomology and serology of isolates of *Potato virus Y* that share the properties with both PVY^{N} and PVY^{O} strain groups. American Potato Journal. 73: 309-315.

Novy RG, Love SL, Corsini DL, Pavek JJ, Whitworth JJ, Mosley AR, James SR, Hane DC, Shock CC, Rykbost KA, Brown CR, Thornton RE and Knowles NR. (2006). Defender: A high-yielding, processing potato cultivar with foliar and tuber resistance to late blight. American Journal of Potato Research. 89: 9-19.

Neiderhauser JS. (1993). International cooperation and the role of potato in feeding the world. American Potato Journal. 70: 385-403.

Nolte P, Whitworth JL, Thornton MK, and McLntosh CS. (2003). Effect of seed borne *Potato virus Y* on performance of Russet Burbank, Russet Norkotah and Shepody potatoes. Plant Disease. 88(3): 248-252.

Phillip N, Alvarez JM and Jonathen LW. (2010). *Potato virus Y* management for the seed potato producer. CIS.1165.

Raccah B, Gal-on A, Eastop VF. (1985). The role of flying aphid vectors in the transmission of *Cucumber mosaic virus* and *Potato virus Y* to peppers in Israel. Annals of Applied Biology.106: 451-460.

Ragsdale DWR, Edward BR, Olson KD and Thaddee MB. (2006). The value of crop borders for management of *Potato virus Y* in seed potatoes. International Association of Agricultural Economists. Annual Meeting. August 12-18. Queensland, Australia.

Ranteria-canett I, Xoconostle-Cazares B, Ruiz-Medrano R. and Rivera-Bustamante RF. (2011). Geminivirus mixed infection on pepper plants: synergistic interaction between PHYVV and PepGMV. Virology Journal. 8: 104.

Read AF and Taylor LH (2001). The ecology of genetically diverse infections. Science. 292: 1099-1102.

Stewart MG and Lampert EP. (2008). Relationship between inoculum density and vector phenology on the incidence of *Potato virus Y* in tobacco. Annals of Applied Biology. 112(2): 303-312.

Singh RP, Valkonen JPT, Gray SM. (2008). Discussion paper: The naming of *Potato virus Y* strains infecting potato. Archives of Virology 153: 1–13.

Sarah S, Ken F, Amy C, Stewart G, Alex C, and RL Groves. (2011). Management of *Potato virus Y* Wisconsin seed potato production.

(http://learningstore.uwex.edu/Assets/pdfs/A3951.pdf)

The Potato Association of America. (2009). http://potatoassociation.org/Industry%20Outreach/varieties/Russets/russet_burbank.html) . Date retrieved. 11/15/2009.

Srinivvasan R. and JM Alvarez. (2007). Effect of mixed viral infections (*Potato virus Y-Potato leafroll virus*) on biology and preference of vectors *Myzus persicae* and *Marcosiphum euphorbiae* (Hemiptera: Aphididae). Journal of Economic Entomology. 100: 646-655.

Stevenson WR, R Loria, France GD, Weingarten DP. Eds. (2001). Compendium of potato diseases, 2nd edition. APS press, St. Paul.

Sufrin-Ringwald T. and Lapidot M. (2011). Characterization of a synergistic interaction between two cucurbit-infecting begomoviruses: *Squash leaf curl virus* and *watermelon chlorotic stunt virus*. Phytopathology, 101(2): 281-289.

Tatieneni S, Graybosch RA, Hein GL, Wegulo SN and French R. (2010). Wheat cultivar-specific disease synergism and alteration of virus accumulation during co-infection with *Wheat streak mosaic virus* and *Triticum mosaic virus*. Phytopathology, 100: 230-238.

The Potato Association of America. (2009). A Professional Society for Advancement of the Potato Industry.(http://potatoassociation.org/Industry%20Outreach/varieties/Russets).date retrieved 11/10/2013.

Vance, VB. (1991). Replication of *Potato virus X* RNA is altered in coinfections with *Potato virus Y*. Virology 182: 486-494.

Viral Zone. Swiss Institute of Bioinformatics. http://viralzone.expasy.org/all_by_species/268). Date retrieved. 11/15/2013.

Warren M, Kruger K and Schoeman AS. (2005). *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV): Literature review for potatoes South Africa. Department of Zoology and Entomology, Faculty of Natural and Agricultural Sciences, University of Pretoria.

Ward CW and Shukla DD (1991). Taxonomy of Potyviruses. Current problems and possible solutions. Intervirology 32: 269-296.

Wintermantel WM, Cortez AA. Anchieta AG., Gulati-Sakhuja A. and Hladky, LL. (2008). Co-infection by two criniviruses alters accumulation of each virus in a host-specific manner and influences efficiency of virus transmission. Phytopathology, 98: 1340-1345.

Ziebell H and Carr JP. (2010). Cross-protection: a century of mystery. Advances in Virus Research. 76: 211-264.

Table 1: Table 1: Cross-reactivity of *Potato virus S* (PVS) and *Potato virus Y* (PVY) using PVY and PVS antisera, respectively, determined with Enzyme-linked Immunosorbent Assay (ELISA)

Virus antigen	**Antiserum**	
	PVS	**PVY**
PVS		
	*0.538	* 0.154
	(0.324 to 0.752)	(0.111 to 0.213)
PVY		
	* 0.125	*3.636
	(0.108 to 0.176)	(3.275 to 3.988)

*Average of four samples

Healthy potato sample: 0.115

Buffer control: 0.096

(The values in parenthesis show the minimum and maximum range of absorbance values from the ELISA assay)

Table 2: Replications for inoculation of Defender plants with PVS and PVY

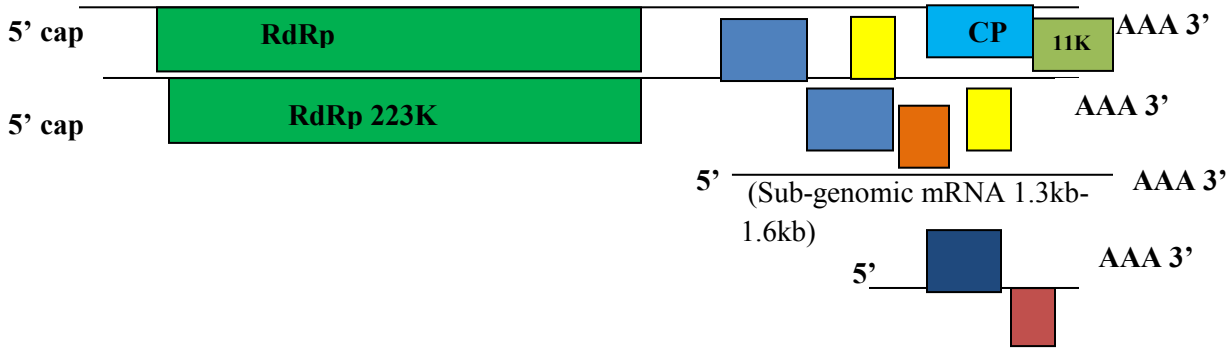
Treatments		
PVS	**PVY**	**PVY + PVS**
Defender	Defender	Defender
Defender	Defender	Defender
Defender	Defender	Defender
Defender	Defender	Defender
Defender	Defender	Defender
Defender	Defender	Defender

Table 3 Replications for inoculation of Desiree plants with PVS and PVY

Treatments		
PVS	**PVY**	**PVY + PVS**
Desiree	Desiree	Desiree
Desiree	Desiree	Desiree
Desiree	Desiree	Desiree
Desiree	Desiree	Desiree
Desiree	Desiree	Desiree
Desiree	Desiree	Desiree

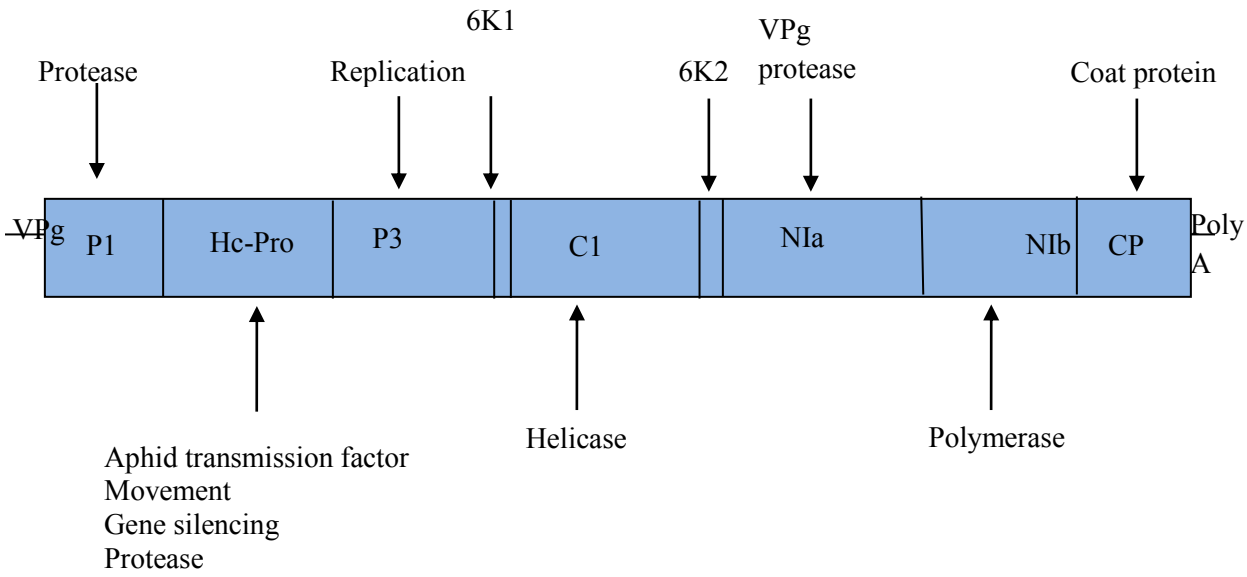
Table 4: Replications for inoculation of Russet Burbank plants with PVS and PVY

Treatments		
PVS	**PVY**	**PVY + PVS**
Russet Burbank	Russet Burbank	Russet Burbank
Russet Burbank	Russet Burbank	Russet Burbank
Russet Burbank	Russet Burbank	Russet Burbank
Russet Burbank	Russet Burbank	Russet Burbank
Russet Burbank	Russet Burbank	Russet Burbank
Russet Burbank	Russet Burbank	Russet Burbank



(http://viralzone.expasy.org/all_by_species/268.html)

Figure 1: Genome organization of a *Carlavirus*. The genome of a *carlavirus* is single-stranded positive sense that has a 5-cap and a plyadenylated tail. It has six open reading frames, and two sub-genomic RNAs.



(Winterhalter, 2005) (http://www.uq.edu.au/vdu/VDUPotyvirus.htm)

Figure 2: Genome organization of *Potato virus Y*: Genome of PVY is single-stranded positive sense RNA genome of 97,000 bases in length.

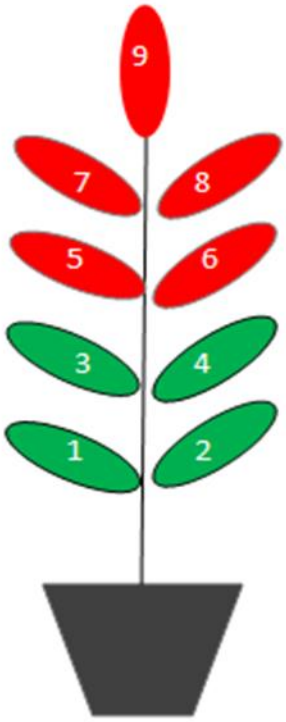


Figure 3: Typical plant diagram showing inoculated leaves (1-4 in green color) and systemic leaves (5-9 in red color).

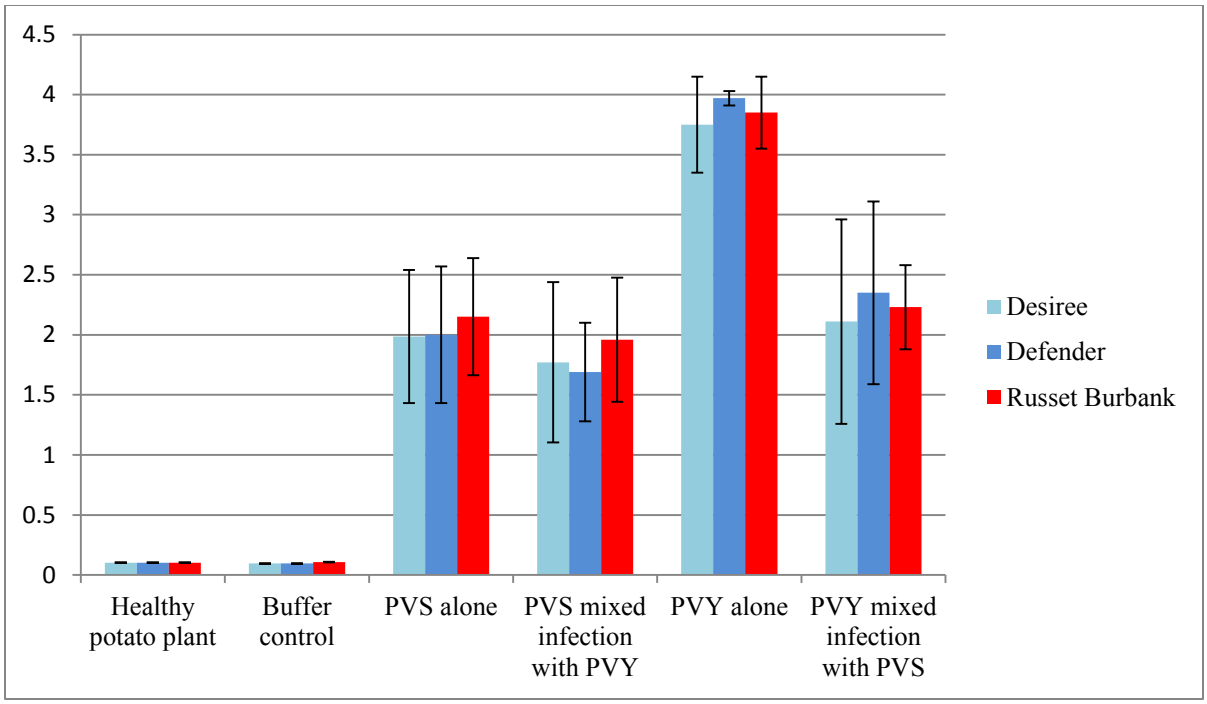


Figure 4: Bar graph showing the results of ELISA of PVS and PVY infected potato plants in single as well as mixed infections. The treatments are shown on the X-axis whereas absorbance (A405) values are shown on the Y-axis. The plants were inoculated with PVS, PVY or both on 03/15/2012 and samples from inoculated plants were harvested 3 weeks post-inoculation on 04/06/2012 followed by ELISA testing on 04/07/2012. Data shown are net absorbance values for mean of the absorbance values of duplicate ELISA wells. The values are shown with standard deviation bar. Healthy potato plant: Uninfected, virus-free potato samples as a negative control; Buffer control: Only buffer was used in place of plant sample; PVS alone: Plants infected with PVS only. PVY alone; Plants infected with PVY only; PVS mixed infection with PVY: Level of PVS in plants doubly infected with PVS and PVY; PVY mixed infection with PVS: Level of PVY in plants doubly infected with PVS and PVY.

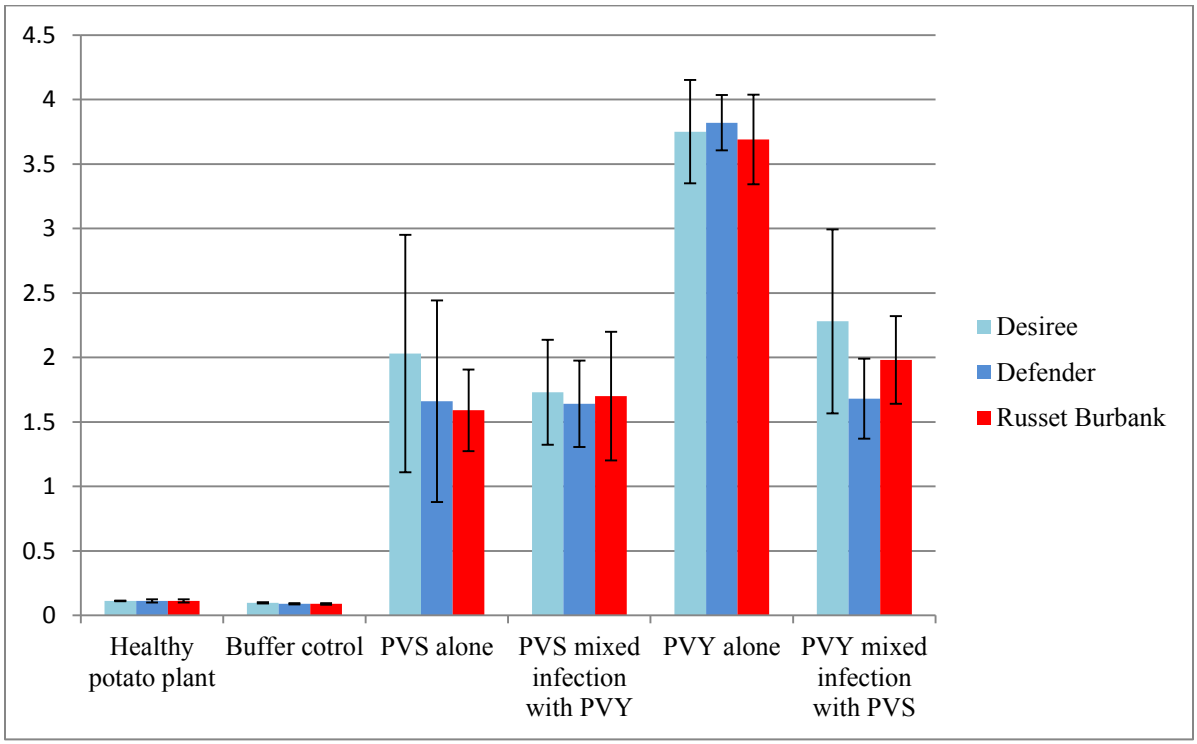


Figure 5: Bar graph showing the results of ELISA of PVS and PVY infected potato plants in single as well as mixed infections. The treatments are shown on the X-axis whereas absorbance (A405) values are shown on the Y-axis. The plants were inoculated with PVS, PVY or both on 10/01/2012 and samples from inoculated plants were harvested 3 weeks post-inoculation on 10/22/2012 followed by ELISA testing on 10/24/2012. Data shown are net absorbance values for mean of the absorbance values of duplicate ELISA wells. The values are shown with standard deviation bar. Healthy potato plant: Uninfected, virus-free potato samples as a negative control; Buffer control: Only buffer was used in place of plant sample; PVS alone: Plants infected with PVS only. PVY alone; Plants infected with PVY only; PVS mixed infection with PVY: Level of PVS in plants doubly infected with PVS and PVY; PVY mixed infection with PVS: Level of PVY in plants doubly infected with PVS and PVY.

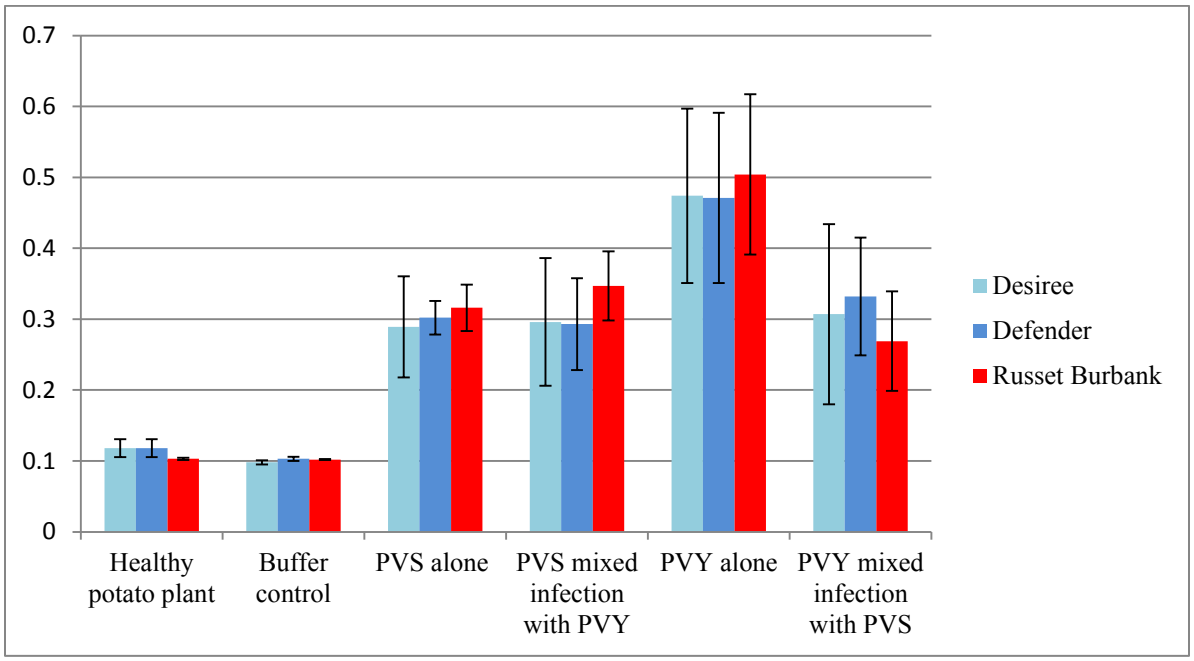


Figure 6: Bar graph showing the results of ELISA of PVS and PVY infected potato plants in single as well as mixed infections. The treatments are shown on the X-axis whereas absorbance (A405) values are shown on the Y-axis. The plants were inoculated with PVS, PVY or both on 01/15/2013 and samples from inoculated plants were harvested 3 weeks post-inoculation on 02/06/2013 followed by ELISA testing on 02/07/2013. Data shown are net absorbance values for mean of the absorbance values of duplicate ELISA wells. The values are shown with standard deviation bar. Healthy potato plant: Uninfected, virus-free potato samples as a negative control; Buffer control: Only buffer was used in place of plant sample; PVS alone: Plants infected with PVS only. PVY alone; Plants infected with PVY only; PVS mixed infection with PVY: Level of PVS in plants doubly infected with PVS and PVY; PVY mixed infection with PVS: Level of PVY in plants doubly infected with PVS and PVY.

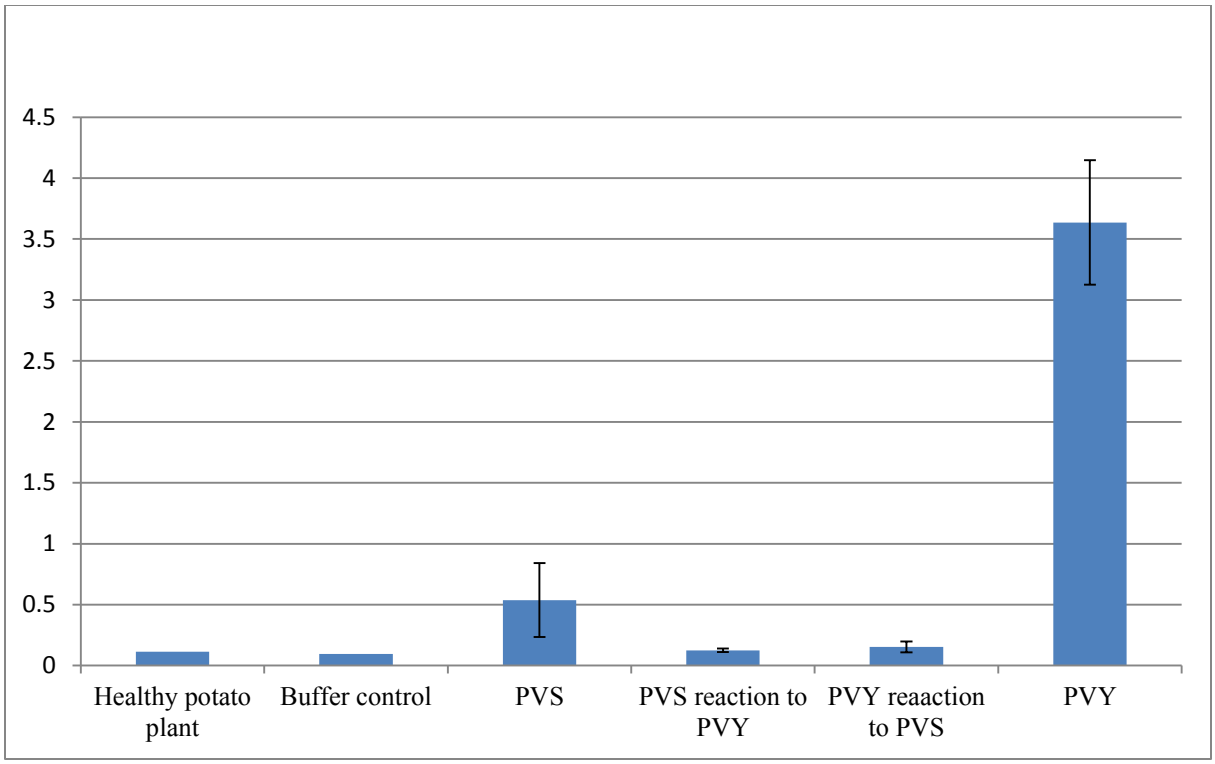


Figure 7: Bar graph showing the results of cross-reaction of PVS and PVY antisera. The sample names are shown on the X-axis and absorbance (A405) values are shown on the Y-axis. Data shown are net absorbance values for mean of the absorbance values of duplicate ELISA wells. The values are shown with standard deviation error bar. Healthy potato plant: Uninfected, virus-free potato samples as a negative control; Buffer control: Only buffer was used in place of plant sample; PVS infected potato plants, PVY infected potato samples, plants infected with PVY only.

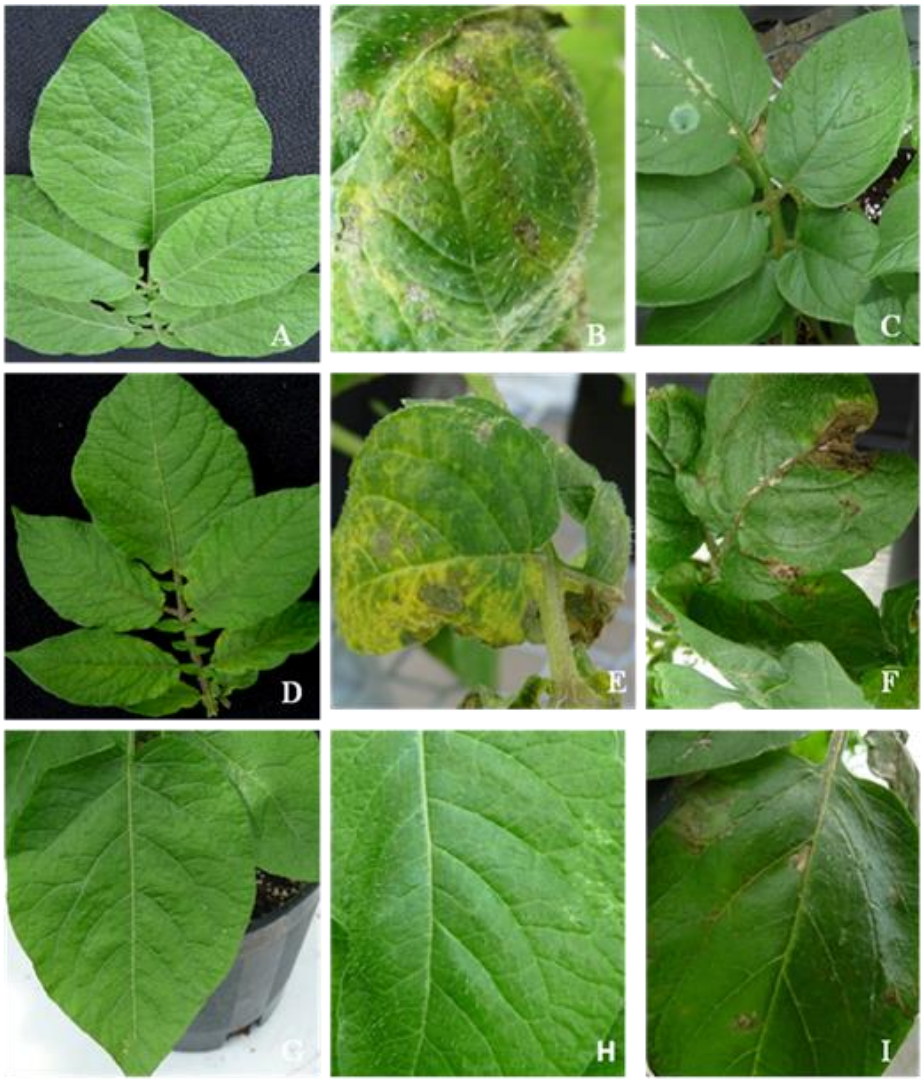


Figure 8: Symptoms of *Potato virus S* (PVS) and *Potato virus Y* (PVY) in different potato genotypes. (A) Symptomless infection of PVS in Russet Burbank; (B) Defender leaves showing necrosis caused by PVS; (C) Symptomless infection of PVS in Desiree; (D) Mosaic and mottling of Russet Burbank leaves as a result of infection with PVY; (E) Defender showing mottling and mosaic due to PVY infection; (F) Systemic necrosis of Desiree with PVY infection; (G) Russet Burbank showing mild mosaic and mottling as a result of mixed infection with PVS and PVY; (H) Mild mosaic and mottling in Defender due to mixed infection of PVS and PVY; (I) Desiree branch showing mild necrosis due to mixed infection of PVS and PVY.

CHAPTER FOUR

BIOLOGICAL CHARACTERIZATION OF HYPERSENSITIVE RESPONSE IN DESIREE TO INFECTION WITH ORDINARY AND NECROTIC STRAINS OF *POTATO VIRUS Y*

INTRODUCTION

Potato virus Y (PVY) is an important virus infecting potato crops wherever they are grown resulting in considerable yield and quality losses (Ward and Shukla, 1991). Potato mosaic disease was separated into X and Y constituents as a result of the experiments in which it was found they the disease was needle and insect transmitted. The needle transmitted component was named as X and aphid transmitted component to tobacco was named as Y. The X component produced concentric rings in tobacco whereas Y component resulted in the development of dark green areas around the leaf veins (Smith, 1931). The ordinary strain of PVY is common in potato fields and it produces mosaic, mottling and stunting in potato. The virus infects economically important plants from nine families including 14 genera of the Solanaceae family; eggplant, tomato, tobacco and potato (Gray *et al*., 2010; Kerlan, 2006). Ordinary strain induces hypersensitive response (HR) in potato carrying Ny gene: Desiree and Maris Bard and they show HR upon infection with ordinary strain of PVY (Mihovilovich *et al*., 1997). Some other PVY isolates elicit HR in potato genotypes carrying the 'Nc' gene such as King Edward and they are referred to as PVY-C isolates. PVY isolates which could overcome these 'Ny' and 'Nc' genes and did not elicit HR in these potato genotypes were named as PVY-N strain group (Cockerham, 1943 and DeBokx, 1961). Stunting and foliar mosaic are typical symptoms of PVY infection in potato. Some potato varieties which include Russet Norkotah, GemStar Russet, Russet Norkotah,

Shepody and Silverton Russet do not show any symptoms of PVY and they are referred to as PVY carriers (Rykbost *et al*, 1999 and Nolte *et al*., 2002).

GENETICS OF DESIREE

Desiree is a main crop potato cultivar that was bred in Netherlands in 1962 as a result of cross of Urgenta and Depesche (tuberosom species). Tubers are large with red skin and yellow flesh. It has high resistance to drought conditions and good resistance to powdery scab. Plants have medium height and are spreading in nature. The tubers have red smooth skin, and are round to oblong with creamy yellow flesh. The vines are early maturing that take 80-100 days and the plants are resistant to resistant to many common potato diseases (The European Cultivated Potato Database, 2013). Plants have evolved efficient ways to prevent the invasion of their tissues by pathogens, and disease is not a routine phenomenon. Rapid development of cell death at and around the infection site, called the hypersensitive response (HR) is a form of disease resistance (Agrios 1988, Goodman and Novacky, 1994). The HR can be triggered by a wide variety of pathogens and occurs locally within few hours following pathogen contact (Mittler, 2007).

RESISTANCE TO PVY IN POTATO

Resistance in potato to PVY infection is divided into two types; hypersensitive response is controlled by dominant 'N' genes and extreme resistance is under the control of 'R' genes. PVY cannot be detected in plants carrying extreme resistance 'R' genes. R gene resistance is considered durable whereas N gene resistance is strain specific and is not durable. Genes for resistance to PVY are present in wild potato species: *S. tuberosum* subsp. Andigena (Ryadg) and *Solanum stoloniforum* (Rysto) (Barker 1996; Mihovilovich *et al*., 1997). Desiree carries Ny gene

that is a single dominant gene and confers hypersensitive response to infection with ordinary strain of PVY. The hypersensitive response (HR) is accompanied with the development of mild mosaic and necrotic spots. Four weeks post-inoculation, the HR spreads throughout the plant and becomes systemic which is characterized by the development of mild mosaic and severe necrosis (Mihovilovich *et al*., 1997). Some other potato cultivars produce HR in response to infection with other strains of PVY like PVY-N and PVY-NTN. The cultivars carrying Nc gene such as King Edward show HR upon infection with PVY-C isolates (Singh *et al*., 2007; Cockerham *et al*., 1970). The HR in Desiree and Maris Bard to infection with ordinary strain of *Potato virus Y* was reviewed and studied by Singh *et al*., (1996).

HYPERSENSITIVE RESPONSE

Hypersensitive response is associated with the development of necrotic spots/lesions so that the pathogen is unable to move further and is restricted in infected areas and the movement of the pathogen to healthy areas is reduced and HR slowly leads to systemic acquired resistance (Freeman, 2003). Plant genomes have R genes which recognize the effectors, this initiates a defense response and the pathogen becomes unable to enter the host. The recognition of an effector by an 'R' gene is called gene-for-gene hypothesis. HR is triggered when avr gene products released by the pathogen are recognized by plant R genes. Studies show that the production of hydrogen peroxide (H_2O_2) during HR increases in infected leaves. The HR is divided into two stages, in the first phase an increased amount of H_2O_2 is produced and in the second phase, infected cells produce reactive oxygen species: superoxide ions, hydrogen peroxide, hydroxyl peroxide and nitrous oxide. Ion efflux and the production of reactive oxygen species results in the death of cells surrounding the area of infection (Heath 2000; Mathews, 2007). Dead cells produced during the HR are deficient in nutrients so the pathogen cannot

survive on infected plant tissues any more (Agrios 1988, Dangle *et al*., 1996). The HR mechanism can be triggered by different fungal, bacterial and viral pathogens and usually happens within few hours after the pathogen comes in contact with host. Pathogen's elicitors are able to trigger HR in plants (Ebel and Cosio, 1994).

Several defense related genes are activated as a result of HR. These genes secrete products including chitinases and phytoalexins which have antimicrobial properties. For example, the enzyme chitinase in bean leaves is a potent inhibitor of fungal growth. The production of ethylene in plants can be triggered by ethylene treatment or by pathogen attack (Schlumbaum *et al.*, 1986). Activation of defense related genes is not specific to plant pathogen interactions because abiotic treatments and physical stresses have been shown to activate them (Brederode *et al.*, 1991). Thus HR is not always dependent on living pathogen, but certain gene products can also trigger HR. Purified phytotoxins behave the same way causing oxidative burst that results in the death of plant cells. In soybean plants, treatment with hydrogen peroxide (H_2O_2) induces cell death similar to HR (Gilchrist, 1997 and Levine *et al*., 1996). Superoxide ions (O^{2-}) damage the plasma membrane resulting in subsequent cell death. Superoxide ion is produced as a result of oxidation of NADPH that is membrane oxidase found in mammalian neutrophils (Groom *et al*., 1996). In a study, the cultured parsley cells were treated with glycoprotein elicitor that resulted in hydroxyl and calcium ion influxes, oxidative burst, activation of defense related genes and accumulation of phytoalexins, changes similar to HR (Nurnberger *et al*., 1997). In another study, it was found that oxidative burst does not always result in cell death. Inoculation of *Nicotiana tabacum* cell suspensions with HR inducing bacterial pathogens resulted in oxidative burst but no HR and cell death were observed. Following inoculation of the tobacco cells, oxidative burst occurred 3 hours post-inoculation but

no cell death was observed. The study concluded that oxidative burst is not always leading to HR and cell death (Glazener *et al*., 1995). The interaction of cowpea with biotrophic fungus *Uromyces vignae* is an example of hypersensitive response. HR is a complex process that involves multiple pathways. In cowpea resistant plants to cow pea rust fungus, the nucleus moved away from the site of fungus penetration. In both resistant cultivars showing HR to *Uromyces vignae*, the protoplast collapse due to HR involved different events. The results showed that different pathways are involved in HR in different resistant cultivars (Skalamera and Heath, 1998). The interaction between *Erysiphe graminis* and barley is another example of HR in plants. Barley plants carrying the *MIa12* gene show HR to infection with powdery mildew fungus (Koga *et al*., 1998).

In this study, I characterized the biological responses of Desiree to infection with ordinary (PVY-O) and necrotic strains of PVY (PVY-N and PVY-NTN). I included Russet Burbank as a control in the study. Russet Burbank produces mosaic, leaf mottling and plant stunting as a result of infection with ordinary strain of PVY.

MATERIALS AND METHODS

POTATO VIRUS Y-ORDINARY AND NECROTIC STRAINS

Three strains of *Potato virus Y*: *Potato virus Y*-ordinary strain (PVY-O), *Potato virus Y*-tobacco veinal-necrotic strain (PVY-N) and *Potato virus Y*-necrosis tuber- necrotic strain (PVY-NTN) were maintained in *Nicotiana tabacum* in insect free greenhouse.

PLANT MATERIALS AND VIRUS INOCULATIONS

Healthy plants of potato cultivar Desiree were grown through tissue culture from virus free potato plants and were transplanted in LC1 potting mixture in insect-free greenhouse. Six-

weeks-old plants were inoculated with virus inocula by grinding the PVY-O, PVY-N and PVY-NTN infected leaves of tobacco (*N. tabacum*) in 0.1 M phosphate buffer (pH 7.2) with 0.4% beta-mercaptoethanol added. Before inoculation with PVY, the plants were tested with Double Antibody Sandwich Enzyme-Linked Immunosorbant Assay (DAS-ELISA) to ensure that they were free of any potyvirus infection. Four-five leaves of healthy plants were dusted with a mixture of carborundum powder-320 mesh and celite (1:1) (Fisher laboratories) and rubbed before inoculation to make slight injuries for successful virus inoculation. Following inoculations plants will be kept in greenhouse. Plants of Russet Burbank were also inoculated as a control and the inoculation was performed the same way.

SYMPTOM OBSERVATION

Inoculated plants were observed for symptom development and the symptoms of infection were recorded. Ten days-post inoculation, two systemic leaves were taken from the inoculated plants and tested by DAS-ELISA by using commercially available kit (Agdia, Elkhart, IN). Healthy un-inoculated potato samples were included in the ELISA assay as negative controls and only buffer will be used as buffer control. Samples were tested in duplicate wells by using the same amount of leaf sample (0.50gm) in 1 ml of extraction buffer. Samples that showed nanometer readings three times higher than healthy control were considered positive. Leaves from *N. tabacum* plants infected with PVY were included as positive controls in the ELISA.

RESULTS

SYMPTOMS

Desiree plants started showing symptoms of mosaic and mottling ten days post-inoculation as shown in figure 1. The symptoms became severe with the passage of time and at 15 days post-inoculation necrotic lesions were seen on the infected plants. First, the development of necrotic spots was only seen on inoculated leaves which later then spread throughout the plants and became systemic. Systemic mosaic became more severe with increasing number of days-post inoculation (Figure 3-4). Plants infected with PVY remained stunted and their height was much less than healthy mock-inoculated plants. Newly emerging leaves remain smaller in size and leaf wrinkling was apparent on systemic leaves. The study showed that HR in Desiree is accompanied with characteristic symptoms development and Desiree plants infected with PVY-O strain can be easily diagnosed in field conditions. With necrotic strains (N and NTN), no symptoms were observed in the inoculated Desiree plants (Figure 5).

ELISA ASSAY

The ELISA data was taken at two time points after inoculation of plants with PVY-O: 10 days post-inoculation and 20 days post-inoculation. Healthy and negative controls were included in the ELISA assay and the samples which showed ELISA values three times higher than positive control were considered positive with PVY infection. Russet Burbank plants were included in the study as a comparison because Russet Burbank does not show hypersensitive response to PVY-O infection and the symptoms are very mild in comparison to Desiree. The ELISA data showed that virus levels were comparatively less in Desiree plants in comparison to Russet Burbank plants (Figures 1-2). Furthermore, an interesting thing was observed in Desiree

plants infected with PVY-O than virus levels increased quickly in Russet Burbank plants in comparison to Desiree plants.

It was found that Russet Burbank plants inoculated with PVY had more multiplication of PVY in comparison to Desiree plants inoculated with PVY at 3 weeks post-inoculation. The virus titers were approximately equal in both cultivars at 2 weeks post-inoculation whereas three weeks post-inoculation; PVY titers were comparatively higher in Russet Burbank plants versus Desiree plants. However, the virus was still able to move systemically in infected Desiree plants.

DISCUSSION

Hypersensitive response is associated with rapid cell death and it is a form of defense governed by resistance genes. Programmed cell death is an active process through which plants get rid of pathogens, unwanted cells, specific structures or organs. Programmed cell death has also a role in plant growth, development and morphogenesis (Heath, 2000; Beers, 1997). During the generation of reactive oxygen species, a wide range of enzymes are involved. These enzymes include copper amine oxidase, xanthine oxidase, NADPH oxidase, oxalate oxidase, peroxidases and falvin oxidases (Koyanagi *et al*., 2000; Mathews, 2007). Disease reaction is genetically controlled and it is dependent on components from both host and the pathogen side. There are reports that Desiree shows HR response to infection with ordinary strain of PVY (Mihovilovich *et al*., 1997). In this study, we studied the biological response of Desiree to infection with ordinary (PVY-O) and necrotic strains (PVY-N and NTN) of *Potato virus Y*. The data showed that Desiree shows HR response to infection with ordinary strain of PVY. The HR is initiated in the form of necrotic spots two weeks post-inoculation and becomes more severe with the passage of time. From this study, it became obvious that Desiree carries 'Ny' gene for HR to ordinary strain and not necrotic strains. The ELISA results showed that PVY titers increased considerably

slowly in Desiree plants inoculated with ordinary strain of PVY whereas with necrotic strains; titers of PVY increased at the same rate as they increased in Russet Burbank plants. The slow replication of PVY-O in Desiree complies with the fact that virus replication slows down in resistant plants (Mihovilovich *et al*., 1997). As Russet Burbank plants were inoculated with ordinary strain of PVY to compare the reaction of both cultivars: One cultivar (Russet Burbank) that does not carry any gene for HR to PVY-O where as other cultivar (Desiree) that has Ny gene for HR to PVY-O strain. Symptoms of HR on infected Desiree plants included mosaic, systemic necrosis and stunting of the entire plants. Growth of plants was retarded with increasing number of days following infection with PVY-O and diseased plants could be identified very easily in the field. Russet Burbank plants inoculated with ordinary strain produced systemic mosaic and mottling as a result of infection with ordinary strain, however, Russet Burbank plants did not show stunting of the entire plants and growth of plants was not affected. Relative virus levels of PVY were measured at two time points following inoculation: 15 days post- inoculation and 21 days post- inoculation in Russet Burbank and Desiree plants. Similarly, the comparative levels of PVY-N and PVY-NTN were determined in inoculated plants at two time points following inoculation: 16 days post-inoculation and 21 days post-inoculation. The ELISA data showed that the relative levels of PVY were similar in plants infected with PVY-N and PVY-NTN. Desiree plants infected with PVY-N and NTN did not show any visible symptoms in contrast to plants infected with ordinary strain of PVY which showed strong hypersensitive response and systemic stunting of entire plants. The study showed that Desiree carries gene for HR to ordinary strain of PVY and not to necrotic strains: N and NTN. It was found that HR was influenced by greenhouse temperatures and the onset of necrotic reaction was delayed when the greenhouse temperatures were high whereas during low greenhouse temperatures below 20^0C, HR was

observed 10-12 days post-inoculation. Some potato cultivars like Yukon Gold and Rywal carry genes Ny-1 for HR to infection with necrotic strains of PVY: N and NTN (Otulak and Garbaczewska., 2012). Following PVY inoculation, local necrosis was observed in inoculated plants three days post-inoculation and PVY particles were identified in epidermis, phloem, and mesophyl cells of inoculated leaves. Through electron microscopy, non-capsid virus particles were found 10 hours post-inoculation and the capsid protein was located on virus particles in inoculated leaves five days post-inoculation by using immunogold labeling method. The inclusion bodies were detected 24 hours post inoculation during HR. Cytopathological changes indicated that cell nucleus may take part in the life cycle of *Potyvirus* virions. During HR to both strains, PVY particles could be detected in the plasmodesmata as well as vascular cells (Otulak and Garbaczewska., 2012). In my study, the hypersensitive response was observed 2-3 weeks post-inoculation depending upon greenhouse temperature and time of the year.

As PVY is going through constant genome alterations due to recombination, resulting in the emergence of new strains, the interaction between certain reported strains and potato cultivars/varieties is important and it is important to learn in detail the changes going on in potato plants infected with different strains of PVY (Visser *et al*., 2012; Chikh *et al*., 2007). In this study, I mainly focused on symptomology of Desiree plants infected with ordinary and necrotic strains and future studies will be focused on analyzing the interaction of Desiree with the three strains at cellular and molecular levels. Studies on early events during disease response will be more interesting and yet they need to be investigated. The study on the early events going on during the interaction of Desiree plants with infection with ordinary and necrotic strains will reveal further steps later in the infection process. In addition to the symptomology, I also determined the rate of multiplication of different PVY strains in Desiree plants at different time

points following inoculation. The different rates of multiplication of ordinary and necrotic strains of PVY in the same potato cultivar indicate that there are certain physiological parameters which influence the multiplication of PVY virions in the same genetic background of potato. These factors can be HR that is present in Desiree towards PVY-O strain or they can be other factors present in Desiree plants: growth stages of Desiree plants, the physiology of Desiree or its reaction towards virulent and a virulent strains of the same virus.

REFERENCES

Agrios GN. (1988). Plant Pathology. London: Academic Press.

Baker H and Harrison BD. (1984). Expression of genes for resistance to *Potato virus Y* in potato plants and protoplasts. Annals of Applied Biology. 105: 539-545.

Baker CJ and Orlandi EW. (1995). Active oxygen in plant pathogenesis. Annual Review of Phytopathology. 33: 299-321.

Barker H. (1996). Inheritance of resistance to *Potato virus Y* and *A* in progeny obtained from potato cultivars containing gene Ry: Evidence for a new gene for extreme resistance to PVA. Theory of Applied Genetics. 93: 710-716.

Bennett M, Gallagher M, Fagg J, Bestwick C, Paul T, Beale M and Mansfield J. (1996). The hypersensitive reaction, membrane damage and accumulation of autofluorescent phenolics in lettuce cells challenged by Bremia lactucae. Plant Journal. 9: 851-865.

Bent AF. (1996). Plant disease resistance genes: function meets structure. Plant Cell. 8: 1757-1771.

Bestwick CS, Bennett MH and Mansfield JW. (1995). Hrp mutant of *Pseudomonas syringae* pv phaseolicola induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in Lettuce. Plant Physiology. 108: 503-516.

Bonfoco E, Krainic D, Ankarcrona M, Nicotera P and Lipton SA. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild or intense insults with N-methyl-D-aspartate or nitric oxide /superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. USA. 92: 7162-7166.

Bradley DJ, Kjellbom and Lamb CJ. (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich cell wall protein: a novel, rapid defense response. Cell 70: 21-30.

Brederode FT, Linthorst HJM, and Bol JF (1991). Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethylene treatment, UV light and wounding. Plant Molecular Biology. 17: 1117-1125.

Bushnell WR. 1981. Incompatibility conditioned by the Mla gene in powdery mildew of barley: the halt in cytoplasmic streaming. Phytopathology, 71: 1062-1066.

BuE schges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van de Lee T, Groenendijk J, ToE psch S, Vos P, Salamini F and Schultze-Lefert P. (1997). The barley Mlo gene: a novel trigger of plant pathogen resistance. Cell 88: 695-705.

Chen Z, Silva H and Klessig DF. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262: 1883-1886.

Chen CY and Heath MC. (1991).Cytological studies of the hypersensitive death of cowpea epidermal cells induced by basidiospores-derived infection by the cowpea rust fungus. Canadian Journal of Botany. 69: 1199-1206.

Cockerham G. (1970). Genetical studies on resistance to *Potato viruses X* and *Y*. Heredity. 25: 309-348.

Dangl JL, Dietrich RA and Richberg MH. (1996). Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell. 8: 1793-1807.

Delaney T, Uknes S, Vernooj B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut- Rella M, Kessmann H, Ward E and Ryals J. (1994). A central role of salicyclic acid in plant disease resistance. Science 266: 1247-1250.

Ebel J and Cosio EG. (1994). Elicitors of plant defense responses. International Review of Cytology. 148: 1-36.

Freeman, S. (2003). Plant defense systems. Chapter 3. Biological Science. Prentice Hall.

Fukuda H. (1997). Programmed cell death during vascular system formation. Cell Death Differentiation. 4: 684-688.

Gilchrist DG. (1997). Mycotoxins reveal connections between plants and animals in apoptosis and ceramide signaling. Cell Death Differentiation. 4: 689-698.

Glazener JA, Orlandi EW, and Baker CJ. (1996). The active oxygen response of cell suspensions to incompatible bacteria is not sufficient to cause hypersensitive cell death. Plant Physiology. 110 (3): 759-763.

Goodman RN and Novacky AJ. (1994). The Hypersensitive reaction in plants to pathogens. A resistance phenomenon. St. Paul, MN: APS Press.

Groom QJ, Torres MA, Fordham-Skelton AP, Hammond-Kosak KE, Robinson NJ, Jones JDG. (1996). *rbohA*, a rice homologue of the mammalian gp^{91phox} respiratory burst oxidase gene. The Plant Journal 10: 515-522.

Hammond-Kosack KE and Jones JDG. (1996). Resistance gene-dependent plant defense responses. Plant Cell. 8: 1773-1791.

Heath MC, Nimchuck ZL. And Xu H. (1997). Plant numclear migrations as indicators of critical interactions between resistant or susceptible cow pea epidermal cells and invasion hypha of the cow pea rust fungus. New Phytopathology. 135: 689-700.

Heath MC. (2000). Hypersensitive response-related death. Plant Molecular Biology. 44(3): 321-34.

He SY, Bauer DW, Collmer A and Beer SV. (1994). Hypersensitive response elicited by *Erwinia amylovora* harpin requires active plant metabolism. Molecular Plant Microbe Interactions. 7: 289-292.

Jones, RAC. (1990). Strain group specific and virus hypersensitive reactions to infection with potyviruses in potato cultivars. Annals of Applied Biology. 117: 93-105.

Karasev AV, Hu X, Brown CJ, Kerlan C, Nikolaeva OV, Crosslin JM, and Gray SM. (2011). Genetic diversity of the ordinary strain of *Potato virus Y* and origin of recombinant strains. Phytopathology. 101: 778-785.

Koga H, Zeyen RJ, Bushnell WR, and Ahlstrand GG. (1998). Hypersensitive cell death, autoflourescence and insoluble silicon accumulation in barley leaf epidermal cells under attack by Erysiphae graminis f. sp. Hordei. Physiological and Molecular Plant Pathology. 32: 395-409.

Levine A, Pennel RI, Alvarez ME, Palmer R and Lamb CJ. (1996). Calcium-mediated apoptosis in plant hypersensitive disease resistance response. Current Biology. 6: 427-437.

Matthews, B. (2007)."The Hypersensitive Response". Agricultural Research Service: Plant Science Institute. The United States Department of Agriculture. Retrieved 2007-01-12.

Mihovilovich E, LF. Salazar, F. Saguma, and MW Bonierbale. (1997). Survey of the durability of the extreme resistance to PVY derived by Solanum tuberosum subsp. Andigena. CIP program Report.

Nolte P, Whitworth JL, Thornton MK, and McIntosh CS. (2003). Effects of seed borne *Potato virus Y* on performance of Russet Burbank, Russet Norkotah, and Shepody potatoes. Plant Disease. 88(3): 248-252.

Nurnberger T, Nennsteil D, Jabs T, Sacks WR, Hahlbrock K and Schell D. (1994). High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell 78: 449-460.

Otulak K and Garbaczewska G. (2012). Ultrastructural events during hypersensitive response of potato cv. Rywal infected with necrotic strains of *Potato virus Y*. Acta Physiology Plantarum. 32(4): 635-644.

Rykbost KA, Hane DC, Hamn PB, Voss R, and Kirby D. (1999). Effects of seedborne *Potato virus Y* on Russet Norkotah performance. American Journal of Potato Research. 76: 91-96.

Schlumbaum A, Mauch F, Vogeli U and Boller T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367.

Singh M, Singh RP. (1996). Nucleotide sequence and genome organization of a Canadian isolate of the common strain of *Potato virus Y* (PVY-O). Canadian Journal of Plant Pathology. 18: 209–214.

Skalamera D and Heath MC. (1998). Changes in the cytoplasm accompanying infection induced nuclear movements and the hypersensitive response in plant cells invaded by rust fungi. The Plant Journal. 16: 191-200.

Smith KM. (1931) Composite nature of certain potato viruses of the mosaic group. Nature 127: 702

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG and Jones JDG. (1995). Molecular genetics of plant disease resistance. Science 268: 661-667.

Visser JC, Bellstedt DU, and Pirie MD. (2012). The recent recombinant evolution of a major crop pathogen, *Potato virus Y*. PLOS ONE. 7(11): e50631.

Table 1: Response of Desiree to infection with Ordinary and necrotic strains of *Potato virus Y*.

PVY strains	Desiree	Russet Burbank
PVY-O	Hypersensitive Response (HR)	S
PVY-N	Susceptible (S)	S
PVY-NTN	S	S

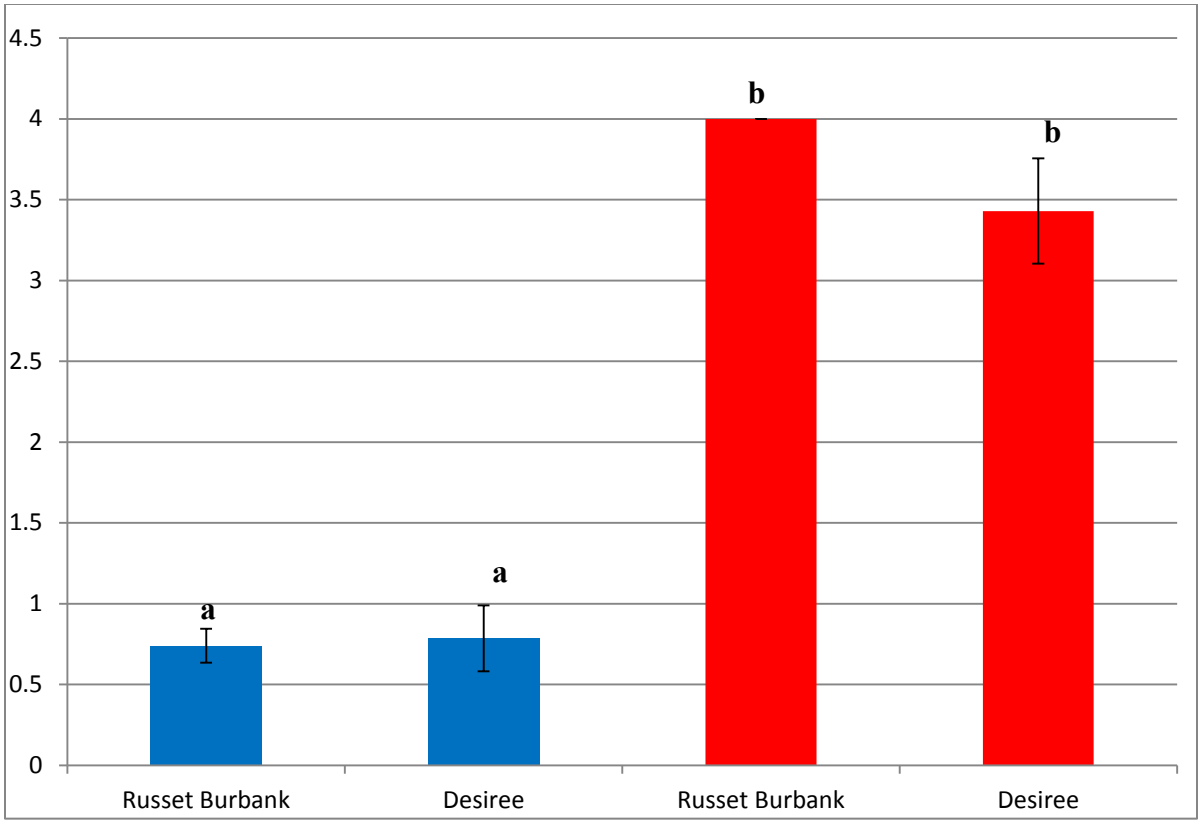


Figure 1: Comparative levels of the ordinary strain of *Potato virus Y* (PVY-O) in Desiree and Russet Burbank plants at two time points following inoculation as determined by ELISA. Red bars indicate virus levels 15 days post-inoculation and blue bars indicate virus levels determined 21 days-post inoculation. Bars with different letters (a,b) denote significant differences at $P<0.05$.

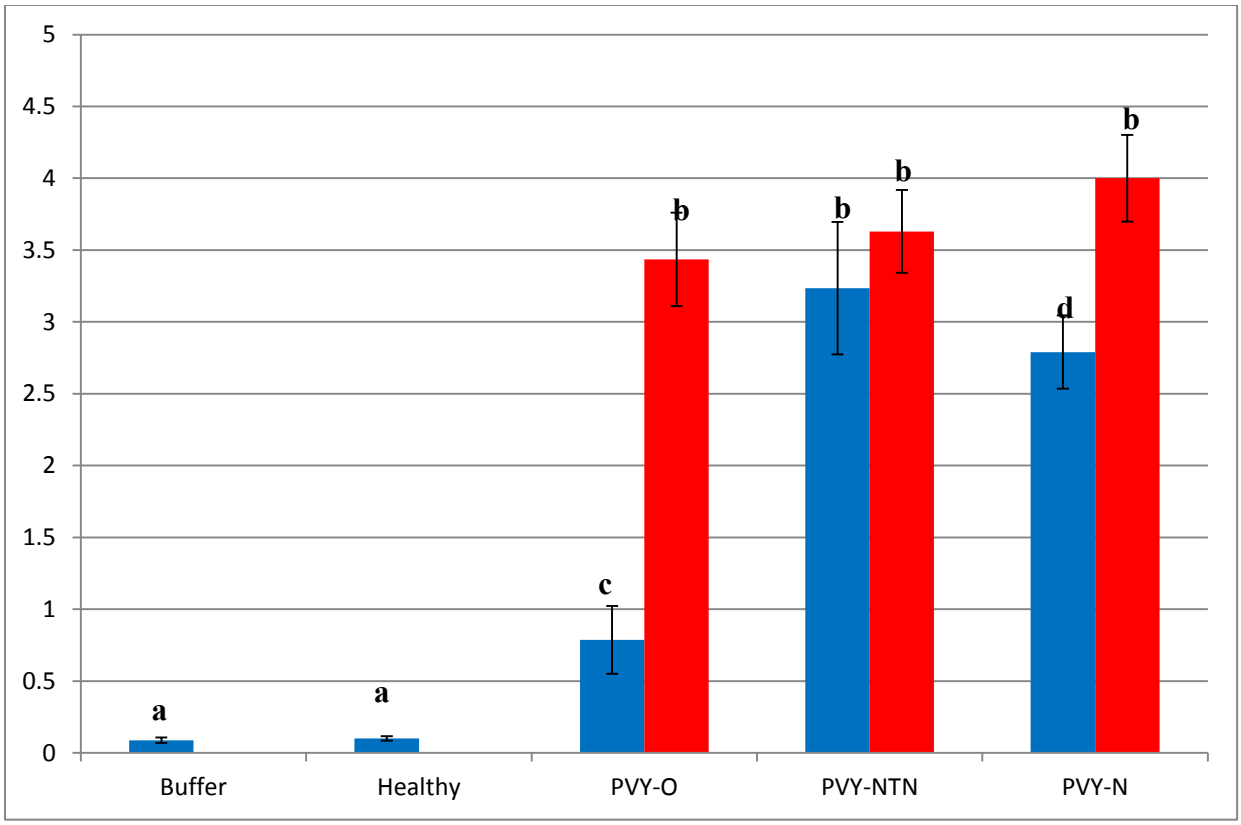


Figure 2: Comparative levels of *Potato virus Y*- ordinary (PVY-O) and necrotic strains (PVY-N and NTN) in Desiree plants 17 days post-inoculation (dark blue bars) and 23 days post-inoculation (light blue bars) as determined by ELISA. Different letters (a,b,c,d) denote significant differences at $P<0.05$.

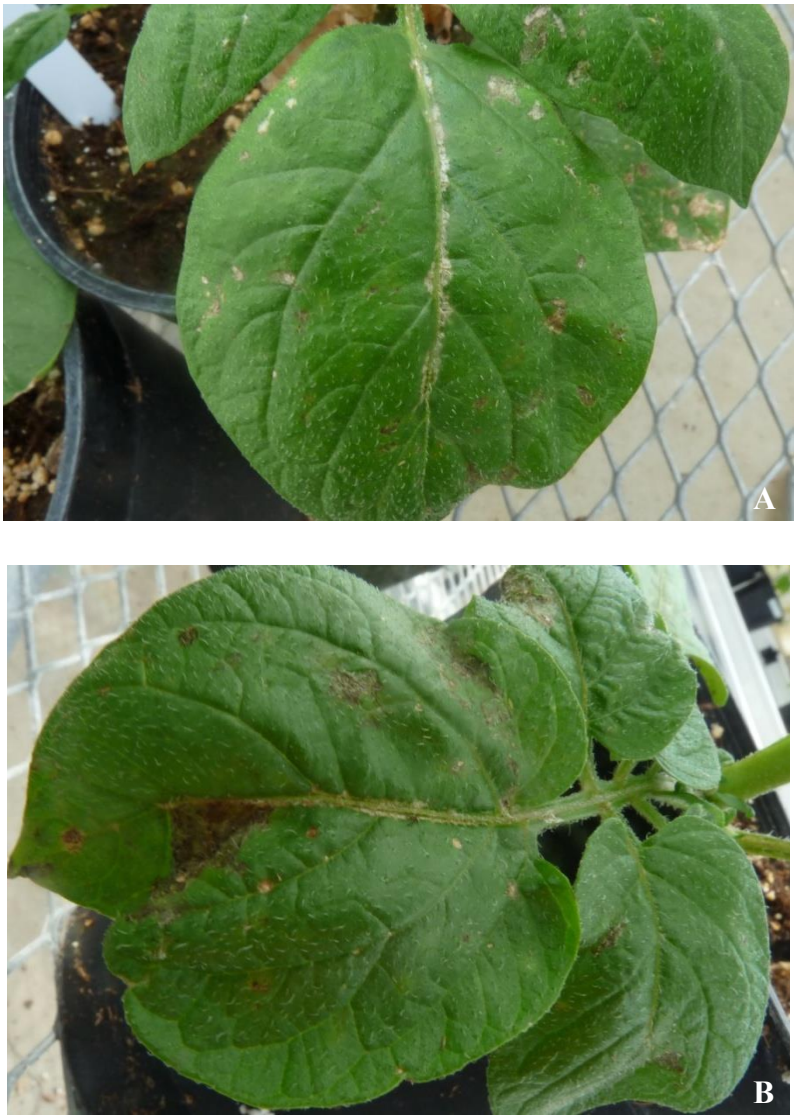


Figure 3: Desiree leaves showing symptoms caused due to infection with ordinary strain of *Potato virus Y*. (A) systemic leaves showing necrotic spots 15 days post-inoculation, (B) systemic leaves showing severe necrosis.

Figure 4: Desiree plants showing symptoms associated with the ordinary strain of *Potato virus Y*. (A) systemic mosaic (B) systemic severe mosaic and mottling and (C) systemic mottling.

Figure 5: *Potato virus Y*-tobacco veinal-necrotic strain (PVY-N) and tuber-necrotic strain (PVY-NTN) do not produce discernible foliar symptoms in Desiree. Above, mechanical inoculation (A) with PVY-NTN (B) and PVY-N 17 days post-inoculation.

CHAPTER FIVE

GENERAL DISCUSSION

Potato virus Y (PVY) is an important pathogen infecting potatoes worldwide causing considerable losses in yield and quality of the produce (Ward and Shukla, 1991). Studies on virus-host interactions have revealed that plants infected with viruses accumulate small RNAs. Detailed understanding about small interfering RNAs (siRNAs) and microRNAs (miRNAs) distribution and expression pattern, abundance and polarity gives useful clues about virus-host interactions (Ding and Voinnet, 2007). The efficacy of RNA-induced gene silencing mechanism depends upon the intrinsic features of viruses and how they interact with the host. Both animal and plant viruses induce the generation of 20-24 nucleotide long siRNAs and miRNAs in infected hosts. During the RNA-interference mechanism, the antisense strand of the siRNA duplex is recruited into the RNA induced silencing complex (RISC complex) and specifically targets and cleaves complementary double-stranded messenger RNAs (Llave, 2010; Burgyan and Havelda, 2011). We reported the small RNA profiles of PVY infected potato plants. This is novel data that provides new information about PVY-derived small interfering RNAs (vsiRNAs) as well as host-derived microRNAs (miRNAs) in infected Russet Burbank plants.

Populations of vsiRNAs in PVY- infected plants were abundant, diverse and derived from every genomic position of PVY. Majority of vsiRNAs were of 20-22 nucleotide classes were derived from the same genomic positions although there were certain hotspots for vsi RNAs regeneration. From PVY-infected Russet Burbank plants, vsiRNAs in size from 20-24 nt were identified. In healthy potato plants, 24 nucleotide class was the most predominant class whereas in PVY- infected potato plants, 21 nucleotide class was the most prevalent class. The

data revealed that the small RNA populations varied between different strains of PVY in infected Russet Burbank plants. The 24 nucleotide class was the predominant class in PVY-infected potato plants with 48.75% of total small RNAs whereas in PVY-infected plants the 21 nucleotide class was predominant: PVY-O (66.46%), PVY-N (47.50%) and PVY-NTN (66.58%). The second most abundant class in PVY- infected plants was 22 nucleotide size class that constituted 12.84% of siRNAs in PVY-O infected plants, 11.52% in PVY-N infected plants and 15.52% in PVY-NTN infected plants. The majority of 21 and 22 nucleotide class small RNAs also highlights the importance of DCL4 and DCL2 enzymes in the RNA-interference mechanism and nullifying these two enzymes will make plants more susceptible to disease (Ding, 2010 and Llave, 2010). The results were in accordance with the results of Ding and Voinnet (2007) where they reported small RNAs in *Arabidopsis* plants infected with positive-stranded RNA viruses belonged to 21 nucleotide class. Abundance of the 21 and 22 nucleotide classes in virus infected plants is also in accordance with the findings of Lin *et al*., (2010) who analyzed virus-derived small RNAs in *Bamboo mosaic virus* infected *Nicotiana Benthamiana* and *Arabidopsis thaliana* plants. The majority of siRNAs were 21-22 nucleotides long and the dominance of 21 and 22 nucleotide size siRNAs in virus-infected plants has been reported for several other plant viruses and it supports the evidence that the 21 nucleotide long siRNA class is the predominant anti-viral silencing component. DCL4 is responsible for generation of 21nt siRNAs in virus infected plants (Blevins *et al.*, 2006; Donaire *et al*., 2009, Ho *et al*., 2007, Qi *et al*., 2009, Molnar *et al*., 2005). Another study that was carried out on viruses infecting grapevines by Pantaleo *et al*., (2010), the prevalent vsiRNAs size was 21 nucleotide corresponding to 65% of total vsiRNAs followed by 22 nucleotide species corresponding to 15% of the total vsiRNAs. *Rice stripe virus* siRNA population in infected rice plants was also dominated by species of 21 (44.8%) and 22 nucleotide

classes (22.8%) (Yan *et al*., 2010). The results indicate that the potato RNA silencing machinery responsible for the biogenesis of endogenous small RNAs tends to produce 24 nucleotide size small RNAs in the absence of the invading pathogen. The maximum population of small RNAs was found in PVY-NTN infected plants (4,957,589) followed by PVY-N (3,887,747) and PVY-O (3,347,434) infected plants. A high population of small RNAs (20-24 nt) in potato plants infected with PVY-N and PVY-NTN was present. Ordinary strain produces systemic mosaic and mottling in Russet Burbank plants whereas PVY-NTN induces chlorotic mosaic and brown spots on tubers (Gray *et al*., 2010; Beczner *et al*., 1984). In our findings, we observed that PVY-O infected potato plants had comparatively less population of vsiRNAs than the plants infected with PVY-NTN and PVY-N. There could be two possible reasons for the differences in the accumulation of virus derived siRNAs in PVY- infected plants. It could be that plants developed a stronger RNA-silencing mechanism towards necrotic strains (N and NTN) which resulted in an increased level of RNA-interference to counteract the pathogen. In comparison to necrotic strains, towards the ordinary strain (PVY-O), the plants showed a weaker gene silencing response, which might have resulted in comparatively less vsiRNAs in PVY-O infected Russet Burbank plants.

VsiRNAs from individual PVY strains represented a genome wide distribution and every genome position was occupied by at least one vsiRNA. The results showed that vsiRNAs were biased towards positive sense strand of the genome in comparison to the negative sense strand. PVY-NTN infected plants had 58.98% vsiRNAs of positive polarity whereas 41.02% vsiRNAs of negative polarity. Likewise, in PVY-N infected plants, 2,577,064 vsiRNAs (66.28%) were derived from plus strand whereas 1,169,143 vsiRNAs (33.72%) were derived from negative strand. Similarly, in PVY-O infected plants, 1,756,935 (52.49%) had positive polarity whereas

590,499 (47.51%) were of negative polarity. The findings are in accordance with the recent findings of Ho *et al*., (2007) and and Qi *et al*., (2009) where according to their results, the more vsiRNAs were derived from plus strand in comparison to the minus strand of genomes of *Turnip mosaic virus* (TuMV) and *Tobacco mosaic virus* (TMV). Another study conducted by Silva *et al.,* (2011) on *Cotton leafroll dwarf virus* (CLDV) reported an equal population of sense versus antisense derived vsiRNAs in virus infected cotton plants. Likewise, preferential accumulation of positive sense vsiRNAs supports a model by which folded RNA within viral ss-RNA serves as a substrate for Dicer (DCL) cleavage (Molnar *et al*., 2005). In our results, there was a strong bias of the sense versus antisense derived vsiRNAs in plants infected with three different strains of PVY. The ratio of sense versus antisense was twice in PVY-NTN infected potato plants whereas in PVY-O and PVY-N infected plants the ratio of sense versus antisense was 1.5: 1. There was the same kind of association of sense versus antisense derived vsiRNAs from different individual genes of PVY-genome except for the P1 gene of PVY- NTN strain where most vsiRNAs came from the antisense strand of the genome. The findings also contrasted with the findings of Li *et al*., (2012) where majority of vsiRNAs were of (-) polarity than those in (+) polarity for potato spindle tuber viroid (PSTVd) in tomato CAHN8. In fact, nearly two thirds (63.6%) of PSTVd siRNAs were in the (-) polarity, with only one third (36.4%) in (+) polarity. Interestingly, in the case of PepMV, the (-) polarity vsiRNAs were also prevalent over the (+) polarity. The findings were in accordance with the findings of Kreuze *et al*., (2009) where they reported the prevalence of positive sense vsiRNAs over negative sense in virus infected sweet potato plants. The data showed that Dicer enzymes are biased in their target cleavage towards the positive-sense RNA strands in comparison to the negative sense strands that resulted in high population of plus-sense derived vsiRNAs. Examination of the relative abundance of sense versus antisense vsiRNAs

from the different RNA fragments of PVY genome showed interesting results. Only in case of NIb gene of PVY-NTN strain, the antisense derived vsiRNAs were more than the sense strand. For all other genes of PVY genome, the number of sense-derived vsiRNAs was greater than antisense derived vsiRNAs. Hotspots of vsiRNAs accumulation are represented by sharp and broad peaks of vsiRNAs abundance scattered throughout the viral genome. The peaks are clusters of multiple reads representing several unique vsiRNAs sequences; sharp peaks denote the presence of abundant reads within the cluster. The pattern of hotspots in plants infected with different PVY strains indicated differences in the presence of hotspots. The hotspots were distributed throughout the viral genome fragments. In addition to vsiRNAs, we also analyzed miRNAs in PVY-infected as well as healthy potato plants. There were considerable differences in the population of unique miRNAs in potato plants infected with three different PVY strains. These results suggested that miRNA expression patterns are being regulated with the virus strain infecting the plants. As these miRNAs have certain roles in plant growth and development as well as defense against invading pathogens and viruses, the production of miRNAs is also being controlled by the different PVY strains (Sunkar *et al*., 2012). All the identified miRNAs belonged to 13 different miRNA families and were expressed differently in different strains.

The study has reported the strainal variation among the vsiRNAs profiles and host derived endogenous small RNAs provides useful clues to understanding the infection mechanism and pathogenicity of PVY. PVY has also been detected in mixed infections with *Potato leafroll virus* (Srinivasan and Alvarez, 2007) and the findings will make it easier to differentiate between multiple viruses infecting the same plant. This is the first report of the vsiRNA profiles of PVY infection of a popular potato cultivar; Russet Burbank. The identification of novel and conserved miRNAs from potato genome will increase the reportier in the miRBase (Griffith-Jones *et al*.,

2007; Griffiths Jones and Kozomara, 2011). Our results can be useful in designing antiviral strategies using RNAi against potyviruses and further understating of symptom expression and silencing suppression with different strains of potyviruses.

Mixed infection of plant viruses is a common phenomenon and has been observed in case of many plant virus diseases. In mixed infections, the viruses may either facilitate each other which is called synergism or they may act against each other resulting in reduced multiplication of each other which is called antagonism (Syller, 2012). We examined the interaction between between *Potato virus S* (PVS) and *Potato virus Y* (PVY) in three commercially grown potato cultivars: Defender, Desiree and Russet Burbank. Symptoms of infection started to develop twoweeks post-inoculation and became severe with the passage of time. In case of PVY, singly infecetd plants symptoms included mosaic, mottling, stunting and wilting of infected plants as well as death of some of infected plants with increasing time post-inoculation. PVS infected plants did not produce symptoms except Defender that showed systemic necrosis and leaf bronzing spots as a result of infection with PVS whereas PVS was symptomless in most potato cultivars. Mixed infection of PVS and PVY resulted in mild PVY symptoms: mottlin and mosaic in ccomparison to plants infected with PVY alone. PVY symptoms were most visible in Russet Burbank which showed systemic mottling, mosaic and plant stunting as a result of infection with ordinary strain of PVY. Desiree plants showed severe systemic necrosis and systemic stunting of plants with PVY infcetion. Symptoms of PVY infection were not visible in Defender which did not show any symptoms of the infection.

Plnats were tested through ELISA by taking systemic leaves from inoculated plants three weeks post-inoculation by using commercially available ELILSA kit (Agdia, Elkhart, IN). Samples were tested in dupliacte wells using commercially available kit (Agdia, Elkhart, IN)

following exactly the kit protocols. Healthy potato samples were included as controls and one buffer control was added as negative control. ELISA data showed that doubly infected plants with PVS and PVY had lower levels of PVY in comparison to single PVY infected plants whereas PVS levels were similar in single and mixed PVS and PVY infections. The OD values for PVS and PVY were similar among three different varieties and this showed that the interaction between PVS and PVY is similar in three different potato cultivars and is not cultivar dependent. Titers of PVS were similar in case of sinlge or double infections with PVY in three cultivars and this showed that PVS replication is not being affectedin Defender, Desiree and Russet Burbank plants. Low levels of PVY in doubly infected plants suggested that PVS is acting against PVY multiplication in doubly infected plantsand has some kind of antagonistic effect on PVY either a phenomenon of cross-protection or mutual exclusion. Whereas PVS levels were simialr in doubly infected plants and singly infected plants with PVS. There were no significant differences in virus titers with respect to three different varieties as illustrated by figures. These findings show that in mixed infections, PVS actsantagonistically towards PVY multiplication.

Virus-virus interactions are host-dependent as well as cultivar dependent as revealed by several exacmples of virus infections in plants. Interaction between PVS and PVY was not affected by three host genotypes and it contrasts with the findings of Gonzalez-Jara *et al*., (2004) in which they found that the interaction between PVY and PVX was host-dependent. There was an enhancement of disease symptoms in *N. tabacum* plants infected with *Potato virus Y* and *Potato virus X*. Synergistic interaction between PVX and PVY led to a 10-fold increase in the titre of PVX compared with single infections (Rochow and Ross, 1955; Vance, 1991). In contrast, no significant increase in PVX levels was recorded in *N. benthamiana* plants co-

infected with PVY, *Tobacco etch virus* (TEV) or *Plum pox virus* (PPV), however, the severe reaction led to systemic necrosis of leaves and stems and finally plant death. The results indicated that the enhancement of disease symptoms is not simply a result of the increase in PVX accumulation in plants. It is found that the synergy pattern between PVX and a potyvirus is host-depedent (Gonzalez-Jara *et al*., 2004). Host-dependent differences in virus accumulation and alteration of accumulation patterns during co-infection, compared with single infections, have also been reported for *Tomato chlorosis virus* (ToCV) In doubly infected *N. benthamiana* plants, TICV titers increased and ToCV titers decreased, when compared with concentrations in singly infected plants, whereas in co-infected *Physalis wrightii* plants, titers of both viruses decreased. The pattern of TICV-ToCV-host interactions suggests the existence of differences between the two viruses in adaptation to different hosts and these differences may finally translate into competetiveness of each virus in doubly-infected hosts (Wintermantel *et al*., 2008). Host-dependent alteration of symptoms has been reported for the plants co-infecetd with *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV), because synergism was observed in *N. tabacum* and *N. benthamiana*, whereas antagonism was found in pepper (Mendez-Lozano *et al*., 2003). A synergy pattern is not only dependent on the host species, but also on the host cultivar, as recently reported for three wheat cultivars co-infected with *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) (Tatineni *et al*., 2010).

HR is associated with rapid cell death is associated with defense governed by resistance genes. Programmed cell death is an active process through which plants get rid of pathogens, unwanted cells, specific structures or organs. Programmed cell death has also a role in plant growth, development and morphogenesis (Fukuda, 1997; Beers, 1997). In other words, HR is correlated with metabolism of plant cells, and it is correlated with the activation of plant

transcription mechanisms which make it different from necrotic cell death that occurs due to physical stimuli or insect damage. Disease reaction is genetically controlled and it is dependent on components from both host and the pathogen side. In this study, we studied the biological response of Desiree to infection with ordinary (PVY-O) and necrotic strains (PVY-N and NTN) of *Potato virus Y*. The data showed that Desiree shows HR response to infection with ordinary strain of PVY. The HR is initiated in the form of necrotic spots two weeks post-inoculation and becomes more severe with the passage of time. The response of Desiree to infection with necrotic strains of PVY: PVY-N and PVY-NTN was carried out in separate experiment. Plants inoculated with necrotic strains did not produce any visible symptoms until 17 days post-inoculation. Plants looked like healthy plants and disease was not visible in inoculated plants. From this study, it became obvious that Desiree carries 'Ny' gene for HR to ordinary strain and not necrotic strains. The ELISA results showed that PVY titers increased considerably slowly in Desiree plants inoculated with ordinary strain of PVY whereas with necrotic strains; titers of PVY increased at the same rate as they increased in Russet Burbank plants.

Russet Burbank plants inoculated with ordinary strain of PVY were compared with Desire plants inoculated with ordinary strain of PVY: One cultivar (Russet Burbank) that does not carry any gene for HR to PVY-O where as other cultivar (Desiree) that has Ny gene for HR to PVY-O strain. Necrotic spots on infected Desiree plants spread systemically with increasing number of days post-inoculation. The HR was characterized with mosaic, systemic necrosis and stunting of the entire plants. Retarded growth was evident in plants showing HR to PVY-O infection with increasing number of days following infection with PVY-O and diseased plants could be identified very easily in the field. Russet Burbank plants inoculated with ordinary strain produced systemic mosaic and mottling as a result of infection with ordinary strain, however,

Russet Burbank plants did not show stunting of the entire plants and growth of plants was not affected. Relative virus levels of PVY were measured at two time points following inoculation: 15 days post-inoculation and 21 days post- inoculation in Russet Burbank and Desiree plants. The data showed that PVY levels were similar in Desiree and Russet Burbank plants at 15 days post-inoculation whereas 21 days post-inoculation the relative levels of PVY were higher in Russet Burbank plants whereas in Desiree plants the relative levels of PVY were comparatively low. Similarly, the comparative levels of PVY-N and PVY-NTN were determined in inoculated plants at two time points following inoculation: 16 days post-inoculation and 21 days post-inoculation. The ELISA data showed that the relative levels of PVY were similar in plants infected with PVY-N and NTN. Desiree plants infected with PVY-N and NTN did not show any visible symptoms in contrast to plants infected with ordinary strain of PVY which showed strong hypersensitive response and systemic stunting of entire plants. The study showed that Desiree carries gene for HR to ordinary strain of PVY and not to necrotic strains: N and NTN. It was found that HR was influenced by greenhouse temperatures and the onset of necrotic reaction was delayed when the greenhouse temperatures were high whereas during low greenhouse temperatures below 20^0C, HR was observed 10-12 days post-inoculation. Some other potato cultivars like Mars Bard carries N gene for HR to infection with ordinary strain of PVY. Some potato cultivars like Yukon Gold and Rywal carry genes Ny-1 for HR to infection with necrotic strains of PVY: N and NTN (Otulak and Garbaczewska., 2010). They analyzed the hypersensitive response of Rywal to infection with necrotic isolates of PVY: The HR was associated with necrosis and PVY particles were detectable in cellular compartments. Certain cytological changes were observed during the HR that indicated that nucleus has a role in the replication of potyviruses. In my study, the hypersensitive response was observed 2-3 weeks

post-inoculation depending upon greenhouse temperature and time of the year. The basic mechanisms going during compatible and incompatible interactions between pathogens and hosts are known but the information about the interaction between virus strains and host varieties is not known. I studied the symptomology of Desiree plants infected with ordinary and necrotic strains and future studies will be focused on analyzing the interaction of Desiree with the three strains at cellular and molecular levels. Studies on early events during disease response will be more interesting and yet they need to be investigated. The study on the early events going on during the interaction of Desiree plants with infection with ordinary and necrotic strains will reveal further steps later in the infection process. In addition to the symptomology, I also determined the rate of multiplication of different PVY strains in Desiree plants at different time points following inoculation. The different rates of multiplication of ordinary and necrotic strains of PVY in the same potato cultivar indicate that there are certain physiological parameters which influence the multiplication of PVY virions in the same genetic background of potato. These factors can be HR that is present in Desiree towards PVY-O strain or they can be other factors present in Desiree plants: growth stages of Desiree plants, the physiology of Desiree or its reaction towards virulent and a virulent strains of the same virus.

REFERENCES:

Blevins T, Rajeswaran R, Shivaprasad PV, Beknazariants D, Si-Ammour A, Park HS, Vazquez F, Robertson D, Meins JF, Hohn T, Pooggin MM. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Research. 34: 6233-6246.

Ding SW. (2010). RNA-based antiviral immunity. Nat. Rev. Immunol. 10: 632–644.

Ding SW and Voinnet O. (2007). Antiviral immunity directed by small RNAs. Cell 130: 413–426.

Donaire L, Wang Y, Gonzalez-Ibeas D, Mayer KF, Aranda MA, Llcave C. (2009). Deep sequencing of plant viral small RNAs reveals effective and widespread targeting of viral genomes. Virology 392: 203-214.

Gonzalez-Jara P, Tenllado F, Martinez-Garcia B, Atencio FA, Barajas D, Vargas M, Diaz-Ruiz J, and Diaz-Ruiz JR. (2004). Host-dependent differences during synergistic infection by *Potyviruses* with *Potato virus X.* Molecular Plant Pathology. 5(1): 29-35.

Griffiths-Jones S., Saini HK., Van Dongen S., and Enright, AJ. (2008). "MiRBase: tools for microRNA genomics". Nucleic Acids Research 36 (Database issue): D154–8.

Ho T, Wang H, Pallet D, Dalmay T. (2007). Evidence for targeting common siRNA hotspots and GC preference by plant Dicer-like proteins. FEBS Lett 581: 3267-3272.

Kreuze JF, Perez A, Untiveros M, Quispe D, Fuentes S, *et al*. (2009) Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. Virology 388: 1–7.

Li R, Gao S, Hernandez AG, Wechter WP, Fei Z, *et al*. (2012) Deep Sequencing of Small RNAs in Tomato for Virus and Viroid Identification and Strain Differentiation. PLoS ONE 7(5): e37127.

Llave, C. (2010). Virus-derived small interfering RNAs at the core of virus-host interactions. Trends in Plant Science. 15: 701-707.

Mendez-Lozano, J., Torres-Pacheco, I., Fauquet, CM., and Rivera-Bustamante, RF. (2003). Interactions between geminiviruses in a naturally occurring mixture: Pepper huasteco virus and Pepper golden mosaic virus. Phytopathology 93:270-277.

Molnar A, Csorba T, Lakatos L, Varallyay E, Lacomme C and Burgyan J. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. Journal of Virology. 79: 7812-7818.

Otulak K and Garbaczewska G. (2012). Ultrastructural events during hypersensitive response of potato cv. Rywal infected with necrotic strains of *Potato virus Y*. Acta Physiology Plantarum. 32(4): 635-644.

Rochow, W. F., and Ross, A. F. (1955). Virus multiplication in plants doubly infected by potato viruses X and Y. Virology 1:10-27.

Sunkar R., Li YF and Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. Trends Plant Sci. 17: 196–203.

Syller J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. Molecular Plant Pathology. 13(4): 204-216.

Tatineni S, Graybosch RA, Hein GL., Wegulo SN, and French R. (2009). Wheat cultivar specific disease synergism and alteration of virus accumulation during co-infection with *Wheat streak mosaic virus* and *Triticum mosaic virus*. Virology. 230-238.

Vance, VB. (1991). Replication of potato virus X RNA is altered in coinfection with potato virus Y. Virology 182: 486-494.

Ward CW and Shukla DD (1991). Taxonomy of Potyviruses. Current problems and possible solutions. Intervirology 32: 269-296.

Wintermantel, W. M., Cortez, A. A., Anchieta, A. G., Gulati-Sakhuja, A.,and Hladky, L. L. (2008). Co-infection by two criniviruses alters accumulation of each virus in a host-specific manner and influences efficiency of virus transmission. Phytopathology 98:1340-1345.

Yan F, Zhang H, Adams MJ, Yang J, Peng J, Antoniw JF, Zhou Y, and Chen J. (2010). Characterization of siRNAs derived from rice stripe virus in infected rice plants by deep sequencing. Archives of Virology. 155(6): 935-40.