CHARACTERIZATION OF RECOMBINANT STRAINS OF POTATO VIRUS Y ASSOCIATED WITH TUBER NECROSIS CIRCULATING IN NORTH AMERICAN POTATOES

A Dissertation

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Authorization to Submit Thesis

This dissertation of Arturo Quintero Ferrer, submitted for the degree of Doctorate in Philosophy with a Major in Plant Science and an orientation in Plant Virology and titled "Characterization of recombinant strains of potato virus Y associated with tuber necrosis circulating in north American potatoes," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Potato virus Y (PVY) is one of the most devastating potato (*Solanum tuberosum*) diseases worldwide costing farmers millions of dollars in losses in yield. PVY exists as a complex set of strains, some with recombinant genomes such as PVY^{NTN} (parental strains PVY^{O} and PVY^{N} causes necrosis in tubers), PVY^{NE-11}(parental strains PVY^{O}, PVY^{N} and an unknown parent causes necrosis in tubers) and PVY^{N-Wi} (parental strains PVY^{O} and PVY^{N} does not cause necrosis in tubers). Pathotype severity is directly correlated to the type of strain that infects the potato and the type of resistance genes, known as N genes, these trigger the hypersensitive response (HR), are strain specific and, potato cultivars have different arrangements of these genes within their genomes. This dissertation will focus on three different projects that were performed in different regions of North America. 1) The first objective was to develop the biological and molecular characterization of hypersensitive reaction genes in two popular Mexican potato cultivars Alpha and Mondial infected with various strains of PVY. 2) The second objective was to investigate the genetic diversity of the NE-11 strain and a novel NTN/NE-11 recombinant of PVY. 3) The third objective was to sequence the full genome of three PVY isolates (Mex 31, Mex 37 and Mex 43) found in a seed potato field and in a wild *Solanum* sp. in the state of Jalisco, Mexico.

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Dedication

I would like to dedicate this thesis to my parents and my sister for their support. If it weren't for them I would not have gotten this far

Table of Contents

List of Tables

List of Figures

Introduction

Plant pathogens such as viruses are present worldwide. Plant viruses can be severely detrimental to the production of agricultural crops including different *Solanum* species such as tobacco, tomato, pepper, and potato. In the case of potato, the most common viruses that have been found in North America are: *Potato virus* S (PVS), *Potato virus* X (PVX), *Potato mop top virus* (PMTV), *Potato virus M* (PVM), *Potato leaf roll virus* (PLRV), *Potato virus* A (PVA), *Tobacco rattle virus* and *Potato virus* Y (PVY). (Halterman *et al*., 2012)

PVY is a member of the family *Potyviridae* and was described for the first time by (Smith, 1931) in potato. PVY encompasses more than twenty five percent of plant viruses, and genus *Potyvirus,* which is the largest genus in the family with at least a hundred species (Adams *et al*., 2011). The virus is a filamentous non enveloped, flexible particle. It's coat protein is used for cell-to-cell movement and protects the positive-sense, single stranded RNA genome of 9.7-kb (Urcuqui-Inchima *et al*., 2001) (95% of plant viruses are composed of positive, single stranded RNA) (Adams *et al*., 2011). The virus replicates in the cytoplasm and generates multiple cytoplasmic inclusions, it encodes a single polyprotein that is later cleaved by virus-specific proteases into ten functional proteins (Urcuqui-Inchima *et al*., 2001). These are: the coat protein (CP) that serves as protection for the virus genome and cell to cell movement within the plant, the helper component proteinase (HC-Pro) is vital for replication, aphid transmission and cell to cell movement, three virus-specific proteinases (P1, HC-Pro, and NIa) that cleave the polyprotein, and virus RNA-dependent RNA polymerase (NIb), and the VPg (viral protein genome-linked) that

replicates the virus and helps it assemble. The cytoplasmic inclusion protein (CI) serves as a helicase, and assists in cell to cell movement by commandeering certain protein channels in the cell wall in plants. It has also been suggested that the CI is a major contributor of plant symptoms and major plant abnormalities such as decreased photosynthetic rate. (Urcuqui-Inchima *et al*., 2001, Hull R. 2009)

Viruses can be transmitted mechanically, through grafting, through potato seed, and by various vectors such as mites, whiteflies, leafhoppers, beetles, fungi, nematodes and aphids (Hull R. 2009). In the case of PVY, transmission into the host can occur in many ways such as grafting, mechanical (plant wounds), plant sap from infected plants and through seed, but the field transmission occurs most commonly by aphids (predominantly the aphid species *Myzus persicae*), in a non-persistent manner (Radcliffe *et al.,* 2002). This means that the aphid can acquire the virus within seconds and can keep the virulent particles in the aphid's stylet for only a short time. Aphids spread PVY in the field in two main ways. Primary spread occurs when infected aphids introduce virus into non-infected field. Secondary spread results when the previously infected plants, serving as reservoirs of the pathogen, provide a source of virus for the progeny left by the first colonizers to spread the virus more extensively into the field. (Hull R. 2009, Radcliffe *et al.,* 2002)

PVY is present worldwide, including North America (Canada, the United States, and Mexico) (Gray *et al*, 2010 and Silva-Rosales *et al*., 2009) and the damage to potato crops alone it amounts to a significant amount of money. The pathogen causes serious reductions in yield by diverting plant metabolism, decreasing the number and size of tubers in the field (Nolte *et al.,* 2004). PVY also

affects tuber quality by inducing a syndrome known as potato tuber necrotic ringspot disease (PTNRD) (Beczner *et al*., 1984 and Le Romancer *et al*., 1994). Not all strains of PVY produce this syndrome. The non-PTNRD group is mostly composed of PVY^{O}, $PVY^{N:O}$, PVY^{N-Wi} and PVY^{C} while the strains that are capable of producing internal necrosis PVY^{Na-N}, PVY^{NTN}, and PVY^{NE11}(Boonham et al.,2002a and 2002b) Nowadays, the predominant strains reported are recombinant viruses such as PVY^{N-Wi}, $PVY^{N:O}$, PVY^{NE-11} and PVY^{NTN} (Singh *et al*., 2008 and Hu *et al*., 2009a).

Plant pathologists use various tools to study and control PVY. Full genome sequencing has proven especially useful to map and compare the evolution of PVY isolates and identify more easily new PVY strains and isolates. This tool has allowed the discovery of many new viruses such as the long and short PVY^{NE-11} (Gudmenstad *et al*., 2008) isolates and different types of PVY^{NTN} such as PVY^{NTNa} and PVY^{NTNb} (Kerlan, 2006). These viruses have very discreet but distinctive arrangements in their genomes, but by comparing them with other known viruses using recombination analysis software, these viruses have been properly classified (Karasev *et al*., 2011).

Another important tool are the genetic studies of resistance genes and their organization in potato. There are two types of resistance genes: extreme resistance genes (ER) and hypersensitive resistance genes (HR). ER genes in plants give them general resistance to all types of PVY, these plants show very limited symptoms. These genes are governed by the R genes and have been found only in wild relatives of potato. (Gebhardt *et al*., 2001)

HR genes are strain specific resistance genes, therefore easier to overcome, but widely available in commercial cultivars and, are governed by the N genes. When

these genes are triggered by a specific strain such as PVY^{O}, the plant produces a biochemical response that we later interpret as symptoms, such as necrosis and mosaic (Tian *et al.,* 2013 and Jones, 1990). These genes can be present in either a homozygous or heterozygous state. Unless this condition is monitored, a resistance gene bred into the plant at one point can be lost over generations if poor plant breeding practices are practiced. That is why plant breeding programs take approximately eight to ten years to produce and ensure a new stable homozygote variety that not only is resistant to pathogens, but keeps or improves the quality of the product (Brown *et al*., 2008).

Both these types of resistance have helped to control certain strains of PVY such as PVY^{O}, PVY^{C} and PVY^{NTN} (Tian *et al.,* 2013). Breeders have worked for many years producing different potato cultivars that have these genes. Unfortunately, other viruses have evolved to overcome the resistance genes of these plants. These plants are said to be “Asymptomatic” and when infected by the virus, they turn into carriers and later contribute to the spread of the virus. It is a good cultural practice to have resistant cultivars in the field and to rogue out other Asymptomatic plants such as weeds and other carrier plants to stop virus spread, but it is more important to keep producing potato cultivars that have better resistance genes (Rowley *et al.,* 2015).

Molecular studies have allowed pathologists to better understand the interaction between the virus and the plant defense mechanisms. Plants do not have antibodies to defend themselves from pathogens, so what they have developed is a mechanism that is able to recognize and eliminate double stranded nucleic acids such as viral RNA. The plant protein DICER (protein with an RNAse domain that

selectively cuts and dices foreign forms of RNA) cuts these RNAS into small pieces in an attempt to stop the replication of the virus (Waterhouse *et al*., 2001). In some cases, these small pieces serve as intercellular signals that move from the top and to the bottom of the plant, and may be instrumental in allowing the apical meristem cells to escape the virus (Thomson, 1956). Chemical signals are also produced; these trigger a localized process of programed cell death, also known as apoptosis, a process that kills virus-infected cells in order to spare uninfected neighboring cells. The cell death is what we macroscopically, interpret as symptoms. But unfortunately for the plant, the virus is sometimes able to shut down the plant's defense mechanisms by producing antisilencing proteins, proteins that block the spread of the small RNAS, and proteins that target key defense proteins to ubiquitination or degradation (Maia,IG *et al*., 1996).

Further study of this biological mechanisms will allow pathologists to understand the rate of a virus infection in the field, allow the pathologist to know if the farmer is using resistant cultivars, and more importantly know if the cultivars used in the field are Asymptomatic cultivars or not.

Biological studies using molecular markers such as GFP, have complemented the previous studies by allowing researchers to observe in real time how the virus infects a plant (Lecellier CH. *et al*., 2004). These studies have been used to identify which tissues the virus predominantly infects in the host, and more importantly, to know if the viruses are phloem limited or not. Certain viruses such as PLRV, are phloem limited, this means that the virus only travels through the vascular system. This is important, since the transmissibility of the virus is directly correlated to the

type of plant tissues the virus is restricted to. PVY for example, can be transmitted in many ways since it is not phloem limited, but PLRV can only be transmitted through vectors like the aphid in a persistent (less efficient) manner (Kerlan C., 2006 and Hull R. 2009).

The use of molecular characterization, specifically the enzyme-linked immunosorbant assay (ELISA) and the reverse transcriptase polymerase chain reaction (RT-PCR), has been important in the prevention of PVY. These techniques use different detection methods and can be used for both qualitative and quantitative analyses. ELISA uses antibodies that bind specifically to virus coat protein epitope (Clark and Adams, 1977). ELISA can detect different viruses and can also employ monoclonal and polyclonal antibodies to distinguish virus strains, and is also less expensive than RT-PCR. ELISA can be performed in various manners, in the case of PVY, The ELISA utilizes three antibodies in total. This is known as TAS-ELISA (the triple antibody sandwich ELISA) (Karasev *et al*., 2010)

The other technique, RT-PCR, uses reverse transcriptase to convert the viral RNA into complementary DNA (cDNA) and then employs specific primers to amplify the DNA fragment many times until sufficient copies are produced to be detected visually in a gel through electrophoresis. So far there have been three main sets of primers developed for the study of PVY (Lorenzen *et al.*, 2006b, Chik-ali *et al.,* 2010). They all detect PVY^{O} and PVY^{N} and certain recombinants.

A better and faster assay known as immunocapture RT-PCR has been developed that encompasses these techniques. This technique uses antibodies to capture the virus, PCR to turn it into cDNA and depending the set of primers used,

can distinguish various strains of PVY. The use of this technique facilities easier detection of new recombinants, confirms infection in Asymptomatic plants, and also allows the detection of coinfected samples (Chik Ali *et al*., 2015).

The last point is important to mention since certain viruses have a synergistic relationships with each other. PVY and PVX, for example, have such a relationship. Plants that are infected by one of these viruses show the regular virus symptoms such as mosaicism, crinkling, and stunting. But when the plant becomes infected with the second virus, the plant symptoms get significantly worse and cause more severe detrimental effects in yield and quality. (Syller and Grupa 2014)

Another molecular technique is the Western blot. This technique is mostly used to specifically categorize and describe virus proteins. When a pathologist needs to study a specific protein that a virus produces, a Western can be performed. This technique can be qualitative and quantitative depending on what the pathologist is looking for (Durrin *et al*., 2010).

An old, but still reliable, technique using the electron microscope has allowed pathologists to discover and differentiate other pathogens from viruses by just observing the microscope. Viruses have many shapes and sizes; some are shaped as rods in the case tobacco mosaic virus (TMV), some are icosahedrons in the case potato leaf roll virus (PLRV) and others are gemminate or twinned headed such is the case of maize streak virus (MSV) and finally some are filamented like in our case with PVY (Adams *et al*., 2011).

In the field, plant pathologists have assisted farmers to produce a clean system of potato production that is able to produce virus free material. It is important

to comment that certain regions might employ slightly different protocols in the general scheme of production but, in general they employ the same following steps (Stark *et al*., 2003):

1. Micropropagation production
2. Seed production
3. Commercial production (fresh or processed potato markets)
4. Storage

The micropropagation production stage is the first level that ensures that the starting material to produce potatoes is pathogen free (Thomson, 1956). Tissue culture labs ensure plantlet quality by testing their product for pathogens such as viruses using PCR, using pathogen free facilities to propagate the plantlets, and using anti-viral chemicals and meristem tip culture to ensure clean material to propagate. (Stark *et al*., 2003)

Seed production starts with clean tissue culture material that is planted in sealed greenhouses. From there, the plants are transferred to a clean field and propagated to produce foundation seed. (Stark *et al*., 2003)

Foundation seed is used to expand production to a certain number of generations (the number of generations varies per region) and finally the commercial producer can either sell the final product to the fresh or the processing market. Potatoes are stored when the enviroment turns unfavorable depending of the weather conditions in the region. Certification procedures are also applied to ensure seed quality (Stark *et al.,* 2003).

In the United States of America, production starts in tissue culture where potatoes are multiplied *in vitro* and tested, mainly for viruses. Potato plants are then planted in sealed greenhouses and the tubers produced are later harvested to be grown and multiplied in the field. Seed growers produce only early generation seed, though even some of them may take a portion of the crop out to G2 or even G3. There are also seed producers who purchase some of the early generation seed and increase it, usually for a single year but sometimes two before selling for commercial production. Most commercial production is G3-G4 (fourth and fifth field generation) but some G2 is also used Certifaction inspections are performed each time a seed lot is grown in the field (Stark *et al*., 2003 and Nolte *et al*., 2009)

Both types of growers have to store their potatoes to keep tubers away from the cold environment and in the early storage period to let the potato skins mature (Stark *et al*., 2003). The most important seed potato producing states in the US are: Idaho, Washington, Wisconsin, North Dakota and Oregon (National potato council). The most prevalent potato cultivars produced in the US. are russet potatoes such as Russet Bourbank.

Potato production In Canada is very similar to the production in the United States. Production is started in tissue culture labs which supply miniplantlets to seed growers who will later provide foundation seed to the commercial farmer. The farmer will either produce potatoes for the fresh market or the processing market. They grow and multiply their initial product up to seven generations. The main potato producing provinces in Canada are: Prince Edward Island, Manitoba, Alberta, New Brunswick, Quebec, and Ontario. The most popular potato cultivars in Canada are Russet

potatoes for fresh market and Atlantic for processing. The potato certification process is performed during the groing seasons and during storage (PEI potato board). Inspectors monitor for the following pathogens: fungi (Late blight, early blight, *Rhizoctonia, Pythium* and *Fusarium*), bacteria (ringrot, softrot), and viruses (PVY, PVX, AMV, PVS, and PLRV) (Canadian inspection agency)

In Mexico, the system is comparable to the systems stablished in the US. and Canada, but there are some important differences because potatoes are produced all year long and cultural practices are different. This, unfortunately, means that pathogen preassure is also present all year long. This makes the certification rules established by SAGARPA and Inifap to be more strict for growers in Mexico than in the US or Canada. Seed potato is imported and then established in greenhouses as foundation seed. This seed is later bought by the commercial grower who can now produce the potatoes either for fresh or processing markets. Commercial growers can multiply their product up to eight generations. Also seed and commercial growers do not require lengthy storage periods due to the lack of harsh winters as in Canada and the US. The most important potato producing states are: Chihuahua, Estado de Mexico, Sonora, Sinaloa, and Nuevo Leon. The most common potato cultivars that are produced in Mexico are: Alpha, Mondial, Fianna, Caesar, Agata, and Atlantic. The most popular potato cultivars for the fresh market are Fianna and Alpha and the most popular potato for the processing market is Atlantic. Certification in Mexico happens all year round and is mostly made by inspectors at random, the inspectors look mostly for pathogens such as fungi (late blight, early blight, *Rhizoctonia*,

Pythium, and *Fusarium*) viruses (PVY, PVX, PLRV, AMV, and PVS) and bacteria (bacterial ringrot, Zebrachip, and Soft Rot).

The research pursued in this dissertation shows, the importance of studying the N genes in different potato cultivars against different PVY strains, understand the genetic diversity of PVY^{NE11}, PVY^{NTN} and PVY^{N} to better categorize the isolates within the strain and make a more accurate characterization, to be able to recognize and properly type novel viruses.

These are the following projects discussed in this dissertation:

1.- Biological and molecular characterization of hypersensitive reaction associated genes in two popular Mexican potato cultivars Alpha and Mondial using various strains of *potato virus* Y.

2.- Genetic diversity of the NE-11 strain *potato virus* Y and a novel PVY NTN/NE11 recombinant.

3.- Full genome sequencing of three potato virus Y isolates found in the commercial field and a wild solanum in the state of Jalisco, Mexico.

Chapter 1: Characterization of strain specific resistance in two Mexican potato cultivars, Alpha and Mondial, against five strains of *Potato virus Y*

In preparation - Forthcoming in 2016- Archives of Virology-under consideration

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Abstract

Alpha and Mondial are two of the most widely grown and consumed potato cultivars in Mexico. These two cultivars produce tubers with white skin and white to light yellow flesh. There are two types of resistance genes: extreme resistance genes (ER) and hypersensitive resistance genes (HR). ER genes in plants give them general resistance to all types of PVY, these plants show very limited symptoms. These genes are governed by the R genes and have been found only in wild relatives of potato. HR genes are strain specific resistance genes, therefore easier to overcome, but widely available in commercial cultivars, these are governed by the N genes. When these genes are triggered by a specific strain such as PVY^{O}, the plant produces a biochemical response that we later interpret as symptoms, such as necrosis and mosaic. Up until now, the hypersensitive reaction in Mexican potato cultivars triggered by N genes against potato virus Y (PVY) strains has not been studied in Mexican potato cultivars. The susceptibility of these cultivars (Alpha and Mondial) to different strains of PVY was tested by mechanically inoculating isolates of the representing strains PVY^{EU-N}, PVY^{Z}-NTN, PVY^{O}, PVY^{NE-11}, and PVY^{N-Wi}. Induction

of a strain-specific, hypersensitive resistance (HR) response relative to two control cultivars, Maris Bard and Desiree were observed. Both Mondial and Alpha displayed HR against PVY^{O} and PVY^{Z}-NTN strains but not against PVY^{Eu-N} and PVY^{N-Wi} strains. Cultivar Mondial was also found to display HR against PVY^{NE11}, while Alpha was fully susceptible to this strain. This is the first study of strain specific resistance against PVY for potato cultivars grown in Mexico, suggesting risks of spread of several recombinant strains of PVY in Mexican potato.

Introduction

Potato production in Mexico happens all year round, predominantly in six states, the state of Chihuahua, Estado de Mexico, Sinaloa, Sonora, Nuevo Leon, and Guanajuato (SAGARPA, 2007). In Mexico, the farmers and consumers have a great preference for cultivars that produce tubers with white to yellow skin and flesh such as Agata, Caesar, Fianna, Fabula, Monalisa, Atlantic, Alpha, and Mondial. The seed growers secure mini-tubers (G0) from private tissue culture companies. Seed farmers grow the potatoes up to a maximum of six generations, and very commonly rotate crops with alfalfa, corn, beans, and sorghum. In Mexico, it is common to clean the tubers at harvest and ship them in this manner to retail stores (SAGARPA, 2007).

Alpha is an old potato cultivar of European origin (Holland), and produces tubers of white to light yellow flesh and white skin. It has high resistance to late blight and potato scab, and medium resistance to certain viruses such as *Potato virus* Y (PVY) (up to this project, resistance of this cultivar to PVY strains is unknown), *Potato virus* X (PVX) and *Potato leaf roll virus* (PLRV). (Potato breeding database). Mondial is a potato cultivar of European origin (Germany). It produces tubers of white to

yellow flesh and white skin. It has low resistance to late blight and medium resistance to certain viruses such as *Potato leaf roll virus* (PLRV), *Tobacco rattle virus* (TRV), and *Potato virus* Y (PVY). (The European cultivated potato database and Potato breeding database)

Potato cultivars grown in Mexico have mild resistance to many diverse viral diseases such as *Potato leaf roll virus* (PLRV), *Potato virus* A (PVA), *Potato virus* M (PVM), *Potato virus* X (PVX), *Tobacco rattle virus* (TRV) and *Potato virus* Y (PVY) (The European cultivated potato database and Potato breeding database).

The main issues with these cultivars is that they are difficult to diagnose through symptoms because asymptomatic plants just serve as reservoirs for vectors, like aphids to spread the virus. This leaves molecular methods such as ELISA and PCR as the only viable options for detection. Undiagnosed virus infected seed is mostly propagated through these infected plants, and also this increases the chances for co-infections between different viruses such as PVY with PVX.

Resistant plants combat pathogens using a mechanism that detects virus proteins and produces a signal that sequesters the infected cell or tissue from the entire system causing a visual reaction (Tian *et al.,* 2013). The reaction to the virus in the plant is a degenerative process and depending on the cultivar, it can take up to 15 days to fully develop virus induced symptoms. These symptoms in the foliage of the plant, range from vein necrosis, green rings and necrotic spots (Rowley *et al.,* 2015). The genes responsible for the resistance in the plant are divided into two types: Extreme Resistance (ER) R genes and Hypersensitive Resistance (HR) N genes. The Hypersensitive Reaction genes are classified so far in potato as *Nc*, *Ny*,

and *Nz* (Jones, 1990). These genes confer the capacity to detect and combat specific strains of PVY by causing HR in potatoes. For example, the potato cultivar Desiree will only elicit an HR when infected by isolates from the strain PVY^{O} (Tian *et al.,* 2013). The Extreme Resistance genes (ER), are mostly known in wild potatoes and are more difficult to breed in commercial potatoes, these genes do not cause a severe reaction as the N genes and react to all general strains of PVY (Gebhardt *et al*., 2001).

PVY consists of genetically distinct strains. These are PVY^{C}, PVY^{O}, PVY^{N}, $PVY^{Z,}$ and PVY^{E} (Galvino-Costa *et al*., 2012b and Karasev *et al.,* 2011) PVY strains have all been classified using potato indicators (potato cultivars such as Maris bard and Desiree, that carry certain N genes to identify specific strains of PVY), serology and full genome sequencing: PVY^{C} is a non-recombinant virus strain that interacts with the HR gene *Nc* encoded in potato indicator cultivar King Edward, and it caused no vein necrosis in tobacco (Gebhardt *et al*., 2001). Nowadays PVY^{C} has migrated away from potato and into pepper, due to the selective pressure from potato indicators (kerlan, 2006). PVY^{O} is a non-recombinant virus strain that interacts with the HR gene *Ny* encoded in potato indicator cultivars Maris Bard and Desiree and it also does not cause vein necrosis in tobacco (Tian *et al*., 2013). PVY^{O} is still common in certain areas like North America but is slowly decreasing in incidence (Gray *et al*., 2010). PVY^{N} is a non-recombinant strain that does not interact with any of the known HR genes, but it causes vein necrosis in tobacco (Hu *et al*., 2009b). PVY^{N} is very rare now in both Europe and North America (Lorenzen *et al*., 2006a). PVY^{Z} is a recombinant consisting mainly of a combination of PVY^{N} and PVY^{O}

genome fragments (Kerlan *et al*., 2011). It elicits HR in potato indicator Maris Bard that carries the *Nz* gene and potato ring spot disease (PTNRD) in other potato indicator cultivars such as Yukon Gold (Quintero-Ferrer *et al*., 2013). This effect on tubers particularly causes millions in loses to the industry. Unfortunately, this strain is common worldwide and is increasing in incidence. Finally, PVY^{E} is a recombinant that is composed of PVY^{O}, PVY^{N} and NE-11 genome segments, it elicits no HR in the presence of known *N* genes, causes no vein necrosis in tobacco, but induces PTNRD in susceptible potato indicator cultivars such as Yukon Gold. It has been infrequently found in Europe and in Latin America (Galvino-Costa *et al*., 2012a).

In this project, The N genes of potato cultivars Alpha and Mondial will be fully characterized using PVY isolates: Mont (USA), M3 (Mexico), Tb60 (USA), N1 (USA), ID20 (USA), NE-11 (USA) and Agata (Mexico) from the University of Idaho collection by comparing a hypersensitive reaction to the previously characterized isolates and comparing their HR reaction with control potato indicator cultivars: Maris Bard (*Nc*, *Ny*, *Nz*) and Desiree (*nc*, *Ny*, *nz*). Finally, to test for systemic infection and to confirm the identity of the inoculum at the end of the experiments, inoculated plants will be subjected to enzyme linked immunosorbant assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) testing and typing. At the end of the experiment, tubers will be harvested and scored for PTNRD or other types of tuber damage to finalize the characterization.

Materials and Methods

Alpha and Mondial, were compared to two previously characterized potato indicator cultivars Maris Bard (*Nc*,*Ny*,*Nz*) and Desiree (*nc*,*Ny*,*nz*) (Jones,1990) to accurately type the HR reaction of the previously untested cultivars. Both Desiree and Maris Bard were maintained as tissue culture plantlets at the Tissue Culture Laboratory of the University of Idaho. Potato cultivar Alpha was provided by the USDA Germplasm Collection (Sturgeon Bay, WI) in form of a tissue culture plantlet, while cultivar Mondial was kindly supplied by Dr. C. Benedict from the Washington State Seed Potato Commission in form of a tuber. Both cultivars were established in tissue culture, subjected to chemical treatment with Ribavirin by Lorie Ewing at the University of Idaho Tissue Culture Laboratory and later, tested by RT-PCR for the presence of major potato viruses, and once found virus-free, were also maintained in the Potato Tissue Culture Laboratory collection. The tissue culture plantlets were planted in triplicates in an insect free growth room at a temperature of 23 to 26°C based on (Rowley *et al.,* 2015).

Plants were mechanically inoculated using carborondum as an abrasive to safely produce entry wounds in the target leaves and to prevent mechanical damage due to the mechanical inoculation, cotton swabs were implemented to minimize damage to the target leaves. Inoculation of potato plants was performed at twenty two days after planting (on average), when plants were at approximately the 10 leaf stage using PVY isolates from the laboratory collection (see the Table 1). These PVY isolates were Mont ($PVY^{Eu\text{-}N}$), M3 (PVY^{Z}-NTN), Tb60 (PVY^{O}), N1 ($PVY^{N\text{-}Wi}$), ID20 ($PVY^{NE\text{-}11}$), NE-11 ($PVY^{NE\text{-}11}$), and AG (PVY^{NTN}). Symptoms were observed and

recorded starting at the fifth day post inoculation (dpi) and recording continued for up to five weeks. After symptoms were recorded, leaf samples of each plant were collected from the upper, non-inoculated part of the plant, and tested by triple-antibody sandwich (TAS) ELISA using four monoclonal antibodies *specific* for PVY^{N}, PVY^{O}, and PVY^{O5} serotypes, and a universal antibody for the general PVY detection using the methodology described previously (Karasev *et al*., 2010; Nikolaeva *et al*., 2012). An immunocapture RT-PCR was used to finalize the strain typing as described (Chikh-Ali et al., 2013), with two different sets of primers (Lorenzen *et al*., 2006b; Chikh-Ali *et al*., 2013) targeting the most common recombinant junctions found in PVY strains. Three weeks after all molecular tests were performed, tubers were harvested and scored for PTNRD symptoms if the complete pathotype was developed (the complete brown ring was formed) the tuber would be scored as positive for PTNRD. All observations were recorded based on (Rowley *et al*., 2015).

All results and observations were analyzed to determine if the plants were truly infected and to assess whether there was cross contamination with different PVY isolates during the experiments. Each experiment consisted of planting from forty eight to sixty plants, four cultivars were used in each experiment (Alpha, Mondial and potato indicator cultivars Maris Bard and Desiree). Plants were distributed in the following manner: There would be twelve to fifteen plants used per cultivar, nine to twelve plants were used for inoculation with three to four different PVY isolates at a time, while the other three plants would be separated from the rest to be used as healthy controls. The experiments were repeated three times based on Rowley *et al*., 2015.

Results

Biological characterization of potato cultivar Alpha

Typical local symptoms exhibited on inoculated leaves of Alpha inoculated with a PVY^{O} isolate at 15 dpi are presented on Fig. 1. The characterization of the HR reaction on cultivar Alpha was monitored throughout the entire development of the disease and scored based on Kerlan, 2006 and Rowley *et al.,* 2015. The progression of the symptoms caused in cultivar Alpha by an HR triggering strain of PVY started with: Green rings that in two to three days turned into necrotic rings, which in turn developed into necrotic lesions that later merged and resulted in necrotic veins and finally in fully necrotized leaves. The HR response in Alpha against PVY^{Z}-NTN isolates M3 and AG developed slower than against PVY^{O}, and took on average 7 more days to exhibit full HR on inoculated leaves (Fig. 2, panels A and B).

Nevertheless, both PVY^{O} and PVY^{Z}-NTN isolates elicited a clear HR response in cultivar Alpha, similar to the HR in a control cultivar Maris Bard (Quintero-Ferrer *et al*., 2014), suggesting the presence of both *Ny* and *Nz* genes in this Mexican cultivar. Isolate Mont ($PVY^{Eu\text{-}N}$) did not induce any visible symptoms in Alpha throughout the 5 weeks of observations even though plants tested positive for systemic PVY infection by ELISA and RT-PCR. When inoculated with PVY isolates N1, ID20, and NE11, cultivar Alpha developed systemic mottling, mosaicism, and some stunting, but no HR response was observed on inoculated leaves or on upper, non-inoculated leaves where virus presence was easily detected by ELISA and RT-PCR. No PTNRD was observed in any of the tubers harvested at the end of each experiment, regardless of the PVY strain used. The reactions of cultivar Alpha displayed upon inoculations with

the six tested isolates are summarized in Table 2 along with reactions observed in control cultivars Desiree and Maris Bard. The genetic background for potato cultivar Alpha deduced based on these tests is also listed in Table 2. We concluded that Alpha has both *Ny* and *Nz* genes, but no other *N* genes that can be triggered by PVY strains such as $PVY^{Eu\text{-}N}$, $PVY^{N\text{-}Wi}$ or $PVY^{NE\text{-}11}$ other than PVY^{O} and PVY^{Z}-NTN.

Biological characterization of potato cultivar Mondial

Cultivar Mondial displayed a substantially faster HR response against either PVY^{O} or PVY^{Z}-NTN isolates so that HR reactions appeared about one week before Alpha plants showed the same extent of HR (see Fig. 3; Table 2). Isolate Mont ($PVY^{Eu\text{-}N}$) did not induce any visible symptoms, although plants tested positive by ELISA and RT-PCR at the end of the experiment. When inoculated with two PVY isolates representing the NE11 strain (ID20 and NE-11), Mondial produced a clear HR reaction on inoculated leaves about 14 dpi, and later induced mild systemic necrosis, but the virus was easily detected in upper, non-inoculated, systemically infected leaves at the end of the experiment. Inoculation with isolate N1 ($PVY^{N\text{-}Wi}$) produced mild mottle and mosaic symptoms, and systemic infection was confirmed by ELISA and RT-PCR. No tubers were found exhibiting PTNRD symptoms. The genetic background for potato cultivar Mondial was concluded to have *Ny* and *Nz* genes conferring HR to PVY^{O} and PVY^{Z}-NTN, and also a hypothetical gene *Nne* proposed by Rowley *et al*. 2015 conferring HR to PVY^{NE11}. The HR reactions for cultivar Mondial against six PVY isolates tested are summarized in Table 2.

Discussion

Hypersensitive resistance genes have been characterized extensively by (Jones 1990, Kerlan *et al.*, 1999, Karasev *et al.* 2011) with potato HR genes being classified into three basic modules (nc,ny,nz). So far, disease responses have been performed using PVY^{N}, PVY^{O}, PVY^{E}, PVY^{Z-}NTN, but not PVY-NE-11 (Lorenzen *et al*., 2008). This project, demonstrates a critical difference in the HR response between cultivars Alpha, Mondial, Maris Bard, and Desiree when inoculated with isolates NE-11 and ID20 (Table1). Mondial showed a clear pattern of hypersensitive reaction symptoms when challenged with isolates M3, Tb60, Agata, NE-11, and ID20. The potato indicator, Maris Bard, reacted the same way as Mondial and the second potato indicator, Desiree, only reacted with Tb60.This would suggest that PVY isolates Agata, NE-11 and ID20 react with the *nZ* gene (Jones, 1990).
Alpha shows a clear pattern of hypersensitive reaction with isolates M3, Tb60 and Agata but it did not show any hypersensitive reaction symptoms when challenged with isolates ID20 and NE-11. This would suggest that PVY^{NE-11} could be classified within the PVY^{N} viruses that do not elicit reactions from any known HR genes.

So when comparing all reaction results, ID20 and NE-11 reacted with cultivars Mondial and Maris Bard, but not with cultivars Alpha and Desiree. This suggests that Mondial and Maris Bard could be carrying a resistance gene previously proposed by Rowley *et al*., 2015 and that cultivars Alpha and Mondial possess the following HR resistance genes: Alpha appears to have (*Ny*,*Nz*) while Mondial carries these HR Genes (*Ny*,*Nz*)

Chapter 2: Genetic diversity of the NE-11 strain of *Potato virus Y* (PVY) and a novel NTN/NE-11 recombinant of PVY

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Abstract

In June 2011, a PVY-positive sample of cultivar Atlantic originating from the Prince Edward Island province of Canada was collected at the Washington State University seed lot trial site in Othello, Wa. and, this isolate was designated PE_17. In January 2012, two PVY-positive samples obtained from the cultivars Alturas and Umatilla Russet were designated as isolates MSU457 and MSU470. These two new isolates came from the winter grow-out test conducted by the state of Montana and were subjected to biological and molecular studies. All three samples displayed the N serotype in ELISA, and while PE_17 was typed as PVY^{NTN}, MSU457 and MSU470 were typed as $PVY^{North\ American\ N\ /NE-11}$ ($PVY^{Na-N/NE-11}$) in RT-PCR. These three isolates were subjected to biological characterization in tobacco and to whole genome sequencing. All three isolates induced vein necrosis in tobacco. The full genome sequencing of the two PVY-positive samples MSU470 and MSU457 were identified as belonging to the NE-11 strain of PVY and to be close in similarity to a reference isolate ID20. PVY^{NE11} has a recombinant genome composed of three segments, the first piece homologous to PVY^{Eu-N}, the second piece homologous to PVY^{Na-N}, and the third piece homologous to an unknown PVY parental sequence. The second

segment coming from the NA-N parent was found to have two distinct lengths in two groups of PVY genomes, typified by isolates NE-11 and ID20, and differing by the position of the first recombinant junction.

PE-17, turned out to be a novel recombinant, carrying a fragment of the NE-11 sequence in the middle of the genome that, otherwise, appeared to be similar to PVY-NTN recombinant. This is the first report of this new recombinant type.

Introduction

Potato virus Y (PVY) is a 9.7 kb long filamentous and flexible positive single-stranded RNA virus. It encodes for a single polyprotein that is cleaved into 10 functional proteins: Proteinase 1 (protease activity, P1), Helper component protein (HC-Pro protease activity, causes hypersensitive reactions, antagonizes gene silencing, and helps with aphid transmission), Proteinase 3 (protease activity, P3), 6k1 (directs replicon to cell), Cytoplasmic Inclusion protein (which has helicase activity and assists in cell to cell movement, CI), Virus encoded protein (cell to cell movement, replication, VPg), 6k2 (directs replicon to cell), Nuclear inclusion protein a (primes RNA replication, Nia), Nuclear inclusion protein b (which contains VPg and RdRP, Nib), Coat Protein (which protects RNA particles). (Urcuqui-Inchima *et al.,* 2001 and Hull 2009)

PVY is capable of infecting a wide range of species of the family *Solanaceae*, including pepper, tobacco, eggplant, tomato and potato. PVY can be transmitted mechanically, through seed and by vectors such as aphids (Kerlan, 2006). The green peach aphid *Myzus persicae* is the one of the main vectors of PVY. In seconds, the aphid acquires the virus from the infected plant and can transmit it into a healthy

plant with its stylet, the virus stays on the stylet and does not enter its digestive system, but through that short period of time the virus is fully infectious (Radcliffe *et al.,* 2002).

PVY can have serious negative effects on potato yield (Nolte *et al*., 2004) it can also produce a severe pathotype known as potato tuber necrotic ringspot disease (PTNRD) which is caused by certain PVY strains such as $PVY^{Eu\text{-}N}$, PVY^{NTN}, $PVY^{NE\text{-}11}$ and $PVY^{Na\text{-}N}$ in susceptible potato cultivars like Yukon Gold (Gebhardt et al., 2001 and Kerlan, 2006). PVY can have synergistic interactions with PVX to cause even more severe damages on quality and yield in potato plants (Syller and Grupa 2014 and Hull, 2009).

PVY exists as diverse molecular strains and some of these strains are recombinant, with genomes composed of two (Hu et al., 2009a) or more parental strains (Galvino-Costa *et al*.,2012) . These strains can be differentiated through serology (Karasev et al, 2010), RT-PCR typing (Lorenzen *et al*., 2006b; Chikh-Ali *et al.,* 2013) and total genome sequencing (Hu *et al.,* 2009a). The main strains are $PVY^{Eu\text{-}N}$ (parental strain), $PVY^{Na\text{-}N}$ (parental strain), PVY^{O} (parental strain), PVY^{NTN} (recombinant strain), $PVY^{N:O}$ (recombinant strain), $PVY^{N\text{-}Wi}$ (recombinant strain), and $PVY^{NE\text{-}11}$ (recombinant strain) (Crosslin et al., 2002, Glais et al., 2002 and Lorenzen et al.,2008).

$PVY^{NE\text{-}11}$ contains pieces of three parental strains $PVY^{Eu\text{-}N}$, $PVY^{Na\text{-}N}$, and an unknown PVY parental strain. $PVY^{NE\text{-}11}$ causes vein necrosis in tobacco and PTNRD in susceptible cultivars of potato. It was originally collected by Gudmestad in Nebraska, in 2004 (Lorenzen *et al*., 2008). $PVY^{NE\text{-}11}$ has increased in incidence

throughout the years alongside PVY^{N-Wi}, but the reason for this is unknown (Karasev and Gray, 2013).

In this project, two new PVY^{NE-11} strains were characterized using serological molecular methods as well as full length genome sequencing. These isolates were named MSU 457 and MSU 470 and represent a different clade of PVY^{NE-11} isolates.

Materials and Methods

Virus Isolates

The PE_17 isolate was collected at the Othello seed lot trial test in June, 2011, and came from an Atlantic seed lot that originated in Prince Edward Island, Canada. The leaf sample displaying mosaic was tested for PVY, and after confirmation of its virus-positive status, was subjected to a molecular and biological characterization. Original leaf samples for MSU457 and MSU470 were collected in the Montana winter grow-out test of 2012 from symptomatic potato plants and provided to us by Dr. N. Zidack (Montana State University). The isolates were collected from potato cultivars Alturas and Umatilla Russet, respectively. Later at the University of Idaho, the three isolates of interest were inoculated in tobacco plants (cultivar Burley) and kept in an insect free environment.

Serological characterization

A triple antibody sandwich (TAS) ELISA was used to test the samples. The ELISA used a polyclonal antibody for detecting all general strains of PVY as described by Karasev *et al.* 2010 and monoclonal antibodies to discern between the specific strains PVY^{N}, $PVY^{O,}$ and PVY^{O5}. The IF5 monoclonal antibody was used for identification for PVY^{N} and the monoclonal antibody SASA-N was used for identification of PVY^{N} and PVY^{NTN}.

RT-PCR and Full Genome Sequencing

Nucleic acid extraction was performed using the Dellaporta method described by (Lorenzen et al, 2006b), the RT-PCR typing was done using the protocol also established by (Lorenzen et al, 2006b). Whole genomic sequencing was produced

using various primer pairs based on Hu *et al*. 2009a with a touch down PCR program that consisted of denaturing cDNA at 95^{o}C for 3min, 7 cycles of 95^{o}C for 30 s, 52^{o}C for 30 s -0.5^{o}C /cycle, 72^{o}C extension for 1 min and then 23 cycles of 95^{o}C for 30 s, 48^{o} C for 30 s, 72^{o}C extension for 1 min, followed by a final extension for 5 min at 72^{o}C. The obtained PCR fragments were purified using the EXOSAP IT kit (Affymetrix, Santa Clara, Ca) for cleaning the PCR products and submitted for sequencing to Genewiz (South field, NJ).

The resulting sequences were assembled using a previously described PVY^{NE-11} sequence (Lorenzen *et al.,* 2008), as a reference sequence with the program Seqman (Lasergene). When the full genome was assembled, the virus genome was aligned with other PVY reference isolates using the program CLUSTALW2 as described by (Thompson *et al*., 1994).

The assembled information was later used for recombination analysis with the program RDP4 Marin *et al.,* 2015. These full genomic sequences were subjected to phylogenetic analysis along with other PVY^{NE-11} isolates. Afterwards all sequences were deposited in Genebank (National Institute of Health)

Results

Biological characterization

All three isolates, PE_17, MSU457, and MSU470 induced distinct vein necrosis when inoculated onto three tobacco plants (cultivar *Burley)* with symptoms becoming visible at about 2 weeks post-inoculation. This necrotic response in tobacco suggested that they represented either a PVY^{N} strain, or one of several recombinants causing vein necrosis in all three infected tobacco plants (see Singh *et al*., 2008; Karasev and Gray, 2013).

Serological characterization

The serotyping performed using the TAS-ELISA for all three isolates indicated positive reactions to a PVY polyclonal antibody (Karasev *et al*., 2010), and also to monoclonal antibodies 1F5 (Agdia, Elkhart, IN) and SASA-N (SASA) used for identification of PVY^{N} and PVY^{NTN}. The monoclonal antibody Mab2 (Agdia, Elkhart, IN) was used as a negative control. These serological results classified all three isolates, PE_17, MSU457, and MSU470, as having serotype N. Results summarized in Table 3.

RT-PCR and whole Genome Sequencing

Initial typing of the three isolates using RT-PCR showed that PE_17 produced bands characteristic of a PVYNTN recombinant, while MSU457 and MSU470 were typed as belonging to the NE-11 strain. Results summarized in Table 4.

The whole genome sequencing of MSU457 and MSU470 isolates confirmed that they were similar to $PVY^{NE\text{-}11}$ isolates. When the alignment (CLUSTAL X2), the recombination analysis (RDP4) and a phylogenetic tree (Fig. 4) were produced, it

was found that MSU457 and MSU470 isolates were closer to the isolate ID20 than to the NE-11 isolate. More detailed inspection of the recombination junctions in both isolates revealed that this was due to the shift in a position of the first breakpoint, position 2,009 for NE-11, and position 2, 220 for ID20, MSU457, and MSU470. These new $PVY^{NE\text{-}11}$ isolates were described as short NA-N viruses alongside the isolate ID20.

The whole genome of the isolate PE-17, however, was found to be distinct from the typical PVY^{NTN} strain. While all three recombinant junctions characteristic of PVY^{NTN} were in place, an additional recombination event was revealed in PE-17 genome (Figure 5). Specifically, an approximately 0.8-kb fragment homologous to the NE-11 sequence from an unknown parent was found in the PE-17 genome using the RDP4 programs. PE-17, thus represents a recombinant between the PVY^{NTN} and PVY^{NE11} strains of the virus. Recombinant structures of all three sequenced isolates, PE-17, MSU457, and MSU470, are presented on Figure 6, side by side with parental genomes.

Discussion

PVY^{NE-11} is a strain of PVY that so far has shown a low profile in the field in comparison with other virus strains such as PVY^{N-Wi}, but it is steadily increasing in incidence due to the evolving nature of the virus (Gray *et al*., 2010). This virus strain causes serious negative effects in potato since it both causes yield reduction and affects tuber quality by producing PTNRD (Piche *et al*., 2004; Lorenzen *et al*., 2008).

PVY^{NE11} has been so far found in potato only in North America (Lorenzen *et al*., 2008; Karasev *et a*l., 2011), and its origin and evolution are not clear at this moment. PVY^{NE-11} isolates currently described in the field have had a greater tendency to be associated with the short Na-N clade of PVY^{NE-11} than the long Na-N clade. Based on this, these two newly described PVY^{NE-11} isolates after the recombination analysis showed that they were more closely associated with isolate ID20 and not with isolate NE-11 (Karasev and Gray, 2013). This shows that the short Na-N PVY^{NE-11} isolates are replacing the long Na-N PVY^{NE-11} isolates and that in the future the short clade could be the more prevalent type of PVY^{NE-11}.

So far, the parental strain that constitutes the majority of the PVY^{NE-11} genome has not being found. It could be that this parental strain did not adapt well to the new cultivars of potatoes that were introduced in the field and therefore disappeared. It could be also that this undiscovered strain has found a niche in an unorthodox host. For this reason it is important to keep track of the evolution of PVY^{NE-11} because the discovery of new PVY strains encourages plant breeders to identify other HR genes and incorporate them in commercial potato stocks. In addition, following changes in

virus sequence could lead to models that predict the evolution of PVY so isolates can be properly diagnosed in the future.

PE-17, on the other hand, represents a completely novel type of PVY recombinant, never found before (Karasev and Gray, 2013). One factor that might have prevented this recombinant type from being uncovered previously is its similarity with the ordinary PVY^{NTN} recombinant. Indeed, in our laboratory assays, this isolate displayed N serotype (Table 3), and produced a band pattern in RT-PCR that was indistinguishable from a typical PVY^{NTN} (Table 4). In order to be able to distinguish the PE-17 recombinant genome, we will need to develop an additional set of specific primers targeting the 0.8-kb segment in the center of the virus genome (Figure 6).

Chapter 3: Full genome sequencing of three *Potato virus Y* isolates found in a seed potato field and a wild *solanum* in Jalisco, Mexico.

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Abstract

In September, 2014, a survey of seed potato fields was conducted in the state of Jalisco, Mexico. Leaf potato plants that showed clear virus symptoms such as crinkling and mosaic were collected. These samples were tested for PVY using PVY immunostrips (Agdia, Elkhart, IN) and positve samples were applied into FTA cards (FTA* commercial name) (Sigma, St. Louis, MO), dried, and transported to the Idaho virology lab for further characterization. Collected fifty samples from potato cultivars Fianna, Fabula, Caesar, some unknown cultivars, and finally from a wild potato plant growing along the field road. Three of the fifty samples were positive for PVY, one each from an unknown red potato cultivar, cultivar Fabula, and from a wild potato. RT-PCR typing suggested that two PVY isolates from potato samples Mex 31 (Unknown red potato cultivar) and Mex37 (cultivar Fabula) were recombinant, and the whole genome sequencing identified them as PVY^{NTN} isolates. The third isolate (Mex43), from a wild potato plant growing as a weed, was identified as a non-recombinant, PVY^{N} isolate by both RT-PCR typing and whole genome sequencing. Phylogenetic analysis of this non-recombinant, PVY^{N} genome placed it in a distinct

branch as a possible ancestral sequence relative to other PVY^{N} genomes found in Europe and in North America. This is the first report of a non-recombinant PVY^{N} found in Mexico, and the first report of a PVY^{N} isolate found in a wild potato plant. The genome of Mex43 isolate may represent an ancestral population of non-recombinant PVY^{N} available in the secondary center of origin of potato (Maldonado R.P. 1990).

Introduction

Potato virus Y (PVY) is a member of the family *potyviridae* and the genus *potyvirus* (Kerlan,2006). PVY is comprised of many diverse parental strains (PVY^{Eu-N}, PVY^{Na-N} and PVY^{O}) and recombinant strains such as (PVY^{NE-11}, PVY^{NTN}, PVY^{E} and PVY^{N-Wi}) (Karasev et al., 2013). These strains are spread worldwide and they all do severe damage in potatoes. Some strains can cause more than yield reduction. They can also produce a severe pathotype known as potato tuber necrotic ringspot disease (PTNRD) (Beczner *et al*., 1984). This pathotype is induced by the following strains PVY^{Na-N}, PVY^{NE-11}, $PVY^{E,}$ and PVY^{NTN}. These strains have certain distribution (Galvino-Costa *et al*., 2012b and Le romancer *et al.,* 1994). For example PVY^{EU-N} is rare in both Europe and North America, PVY^{E} is rare in South America and in Europe (Galvino-Costa *et al*., 2012b), PVY^{O} is rare in Europe but quite prevalent in North America, while recombinant isolates like PVY^{NE-11}, PVY^{NTN} , $PVY^{N:O}$ and PVY^{N-Wi} are increasing in prevalence world-wide (Karasev and Gray *et al 2013).*

The recombinant strains PVY^{NTN}, $PVY^{N-Wi,}$ and $PVY^{N:O}$ appear to be primarily derived from PVY^{O} and PVY^{Eu-N} (Singh et al., 2008; Karasev and Gray, 2013). The recombinant strain PVY^{NE-11}, is comprised of PVY strains PVY^{Na-N}, PVY^{Eu-N} and an

unknown parent (Lorenzen et al., 2008). Finally, the recombinant strain PVY^{E} appears to be derived from three parental strains: PVY^{EU-N}, an unknown parent and PVY^{O} (Galvino-Costa *et a*l., 2012b).

Strain composition of PVY in Mexico has not been studied extensively in the past, but PVY it has been spotted before (Flores *et al., 2002,* Ramírez-Rodríguez *et al*., 2009 and Robles-Hernandez *et al*., 2010*)*. Recombinant strains were reported to occur in Mexico, including PVY^{NTN} and PVY^{N-Wi} (Robles-Hernandez et al., 2010; Quintero-Ferrer and Karasev, 2013; Quintero-Ferrer et al., 2014), however, no non-recombinant PVY isolates were described so far. Only one whole genome sequence was determined (Quintero-Ferrer et al., 2014) for a PVY^{NTN} recombinant found in Chihuahua, Mexico, in a seed potato field. This lack of information on the strain composition of PVY circulating in Mexican potato is a serious obstacle in management of PVY in the country.

It is important to know that the climate conditions in Mexico allow the growers to produce potatoes year round (SAGARPA, 2007) and unfortunately alternative hosts for PVY are also available year round. For this reason, farmers double their scouting efforts for vectors such as aphids and applying insecticides and fungicides above recommended usage. In some states of Mexico there is little to no use of irrigation, mostly because production dependent on rain-fall. Farmers plant their potatoes in these high precipitation areas and as soon as the rain season ends, harvest their potatoes and follow the rain to the next state with the favorable conditions. This practice has certain economic advantages for the grower, but unfortunately helps to spread pathogens such as PVY when contaminated material is

transported across the country. Farmers are aware of this issue but find that it is still more profitable for them to risk contaminated material coming in contact with their fields than to pay for irrigation (Personal communication Javier Ireta Moreno).

This project focuses on a detailed molecular study of three isolates of PVY collected during a potato seed survey conducted in the State of Jalisco, Mexico in 2014. The first non-recombinant PVY^N isolate was found in a wild potato plant growing close to a potato field. This PVY^N sequence may represent an ancestral genome circulating in Mexico.

Materials and Methods

Sample collection and virus testing

Fifty leaf samples were collected in the state of Jalisco in 2014 from potato plants exhibiting mosaic and mottling. A single non-symptomatic wild potato plant was sampled next to the potato field where it grew as a weed along the ditch on the edge of the road. All samples were tested for the presence of PVY using immunostrips (Agdia, Elkhart, IN), and those three found positive were pressed onto FTA cards (Sigma, St. Louis, MO), dried, and transported to the University of Idaho for further testing.

RT-PCR and whole Genome Sequencing

Nucleic acid extraction from FTA cards was performed using the methodology described by Ndunguru *et al.,* 2005. Whole genome sequencing was conducted using overlapping RT-PCR fragments directly, as described by Hu *et al.,* 2009b. The touch down PCR program used to run these primers ran in the following manner: Denaturing cDNA at 95ºC for 3min, 7 cycles of 95ºC for 30 s, 52ºC for 30 s -0.5ºC /cycle, 72ºC extension for 1 min and then 23 cycles of 95ºC for 30 s, 48º C for 30 s, 72ºC extension for 1 min, followed by a final extension for 5 min at 72ºC. The obtained PCR fragments were purified using the EXOSAP IT (Affymetrix, Santa Clara, Ca) kit for cleaning the PCR products and submitted for sequencing to the company Genewiz (South field, NJ). The resulting PCR products were assembled using previously described PVY^{NTN} isolates stored in the University of Idaho PVY collection as reference sequences with the program Seqman (Lasergene). When the

full genomes were assembled, the viruses were aligned with other PVY reference isolates using the program CLUSTALW2 (Thompson et al., 1994)

The assembled information was later submitted to recombination analysis using the program RDP4 (Martin *et al.,* 2015). These full genomic sequences were used to generate a phylogenetic tree along (Saitou *et al*., 1987).

Results

RT-PCR and Full Genome Sequencing

The first set of multiplex primers (Lorenzen *et al*., 2006b) for the samples extracted from a red unknown potato cultivar and Fabula both showed a 181bp band and a 457bp band and for the wild potato sample 328bp and 398bp bands. In the case of the other set of primers (Chikh-Ali et al., 2013) for the samples red unknown cultivar and Fabula both showed a 441bp band, a 633bp band and finally a 1307bp band and for the wild potato sample a 398bp, a 633bp and a 1307bp band. The results categorize the red unknown potato cultivar and Fabula cultivars as PVY^{NTN} isolates and the wild potato sample as a $PVY^{EU\text{-}N}$ isolate. These isolates were later designated as Mex31 (unknown red), Mex37 (Fabula), and Mex43 (Wild potato). The results are summarized in Table 5.

The full genome sequencing of the three isolates confirmed that Mex 31 and Mex 37 were very similar to the recombinant strain PVY^{NTN} and that isolate Mex 43 was very similar to the parental strain $PVY^{EU\text{-}N}$. When the alignment (CLUSTAL X2), the recombination analysis (RDP4) and a phylogenetic tree (Fig Tree v1.3.1) were produced. The phylogenetic analysis is shown in Figure 7. Interestingly, the genome of Mex43 was placed separately from either European or North American isolates from the same PVYN strain, and this position suggested a possible ancestral role for this Mex43 sequence (Figure 7).

Discussion

Although recombinant isolates of PVY were reported from Mexico before (Robles-Hernandez et al., 2010; Quintero-Ferrer and Karasev, 2013; Quintero-Ferrer et al., 2014), no reports of non-recombinant strains of PVY have been published. Only PVY^{NTN} and $PVY^{N\text{-}Wi}$ were found in potato in different states of Mexico. Until now, it seemed that the diversity of PVY strains circulating in Mexican potato was limited. This contrasted to situations in other potato-growing areas of the world, like Europe, North America, Brazil, Japan, and Middle East.

Here, we report on an unusual, non-recombinant PVY^{N} isolate found in the state of Jalisco which expands the diversity of PVY circulating in Mexico. Characteristically, this PVY^{N} isolate, named Mex43, appeared to have a distinct sequence, distinguishing it from European and North American isolates belonging to the same strain PVY^{N} (Figure 7). Due to this phylogenetic position, Mex43 can be concluded to have an independent origin, and not be a progeny of PVY^{N} brought from either Europe or North America. We hypothesize that Mex43 may represent an ancestral sequence relative to European and North American PVY^{N}, given its presence in the wild potato sample. If this is true, the PVY isolates circulating in wild potato in Jalisco need serious attention and more thorough study. We are planning the survey of the wild potato in Jalisco, Mexico, for the presence of PVY isolates.

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Table 1. Interactions between hypersensitive reaction genes in potato cultivars Alpha and Mondial using various *Potato virus Y* strains.

List of the PVY isolates used in chapter 1, with their descriptions.

Isolate Name	Strain	Origin	GenBank Accession
Tb60	O	U.S.A.	EF026074
M3	Z-NTN	Mexico	KF850513
AG	NTN	Mexico	Unpublished
Mont	Eu-N	U.S.A.	AY884983
N1	N-Wi	U.S.A.	HQ912863
NE-11	NE-11	U.S.A.	DQ157180
ID20	NE-11	U.S.A.	HQ912867

[a)] Note: Sequence alignment length: 3,133; in all events, p-value was <0.0001

[b)] NR, non-recombinant

Table 2 Genetic typing of various PVY isolates on selected potato cultivar indicators and on tobacco

Hypersensitive Reaction genes (N genes) in potato cultivars Alpha, Mondial, Desiree, Maris Bard and vein necrosis reaction on tobacco cultivar Burley, against six isolate representing four *Potato virus Y* strains.

Strain	**PVY^{O}**	**PVY^{Z}**		**PVY^{N}**	**PVY^{NE-11}**	
Isolate name and genetic background	**Tb60**	**M3**	**AG**	**Mont**	**NE-11**	**ID20**
Alpha (*Ny:Nz*)	**HR**[a]	**HR**	**HR**	S	S	S
Mondial (*Ny:Nz*)	**HR**	**HR**	**HR**	S	**HR**	**HR**
Desiree (*Ny:nz*)	**HR**	S	S	S	S	S
Maris Bard (*Ny:Nz*)	**HR**	**HR**	**HR**	S	**HR**	S
Tobacco (cv. Burley)[c]	S[b]	S	**VN**[d]	**VN**	**VN**	**VN**

[a)] HR, hypersensitive resistance

[b)] S, susceptible

[c)] Reaction on *N. tabacum* cv Burley

[d)] VN, vein necrosis in tobacco

Table 3 Characterization of PVY NE-11 isolates MSU_457 and MSU_470 using TAS ELISA with multiple antibodies.

Molecular characterization of *Potato virus* Y NE-11 isolates PE_17, MSU_457 and MSU_470 using triple antibody sandwich in the enzyme linked immunosorbant assay (ELISA) with multiple antibodies.

Antibodies	**Pab**	**Mab2**	**1F5**	**SASA-N**
PE_17	**+**[a]	**-**[b]	+	+
MSU457	**+**	**-**	**+**	+
MSU470	+	-	+	+
Tb60 (PVY isolate control)	**+**	+	-	-
ME-173 (PVY isolate control)	+	+	+	**-**
N1 (PVY isolate control)	**+**	+	-	-
HR1 (PVY isolate control)	**+**	-	+	+

[a)] Positive by ELISA

[b)] Negative by ELISA

Table 4 Characterization of PVY^{NE11} isolates MSU_457 and MSU_470 using RT-PCR with two sets or multiplex primers

Characterization of PVY^{NE11} isolates PE_17, MSU_457, and MSU_470 using reverse transcriptase polymerase chain reaction (RT-PCR) with two sets of multiplex primers (Lorenzen *et al.*, 2006 and Chikh-Ali *et al*., 2010).

Primer set	**Lorenzen**	**Lorenzen**	**Chikh-Ali**	**Chikh-Ali**	**Chikh-Ali**
PE-17 (Atlantic)	**181 bp[a]**	**452 bp**	**Nt** [c]	**Nt**	**nt**
MSU457 (Alturas)	**328 bp**	**-** [b]	**-**	**633 bp**	-
MSU470 (Umatilla)	**328 bp**	**-**	-	**633 bp**	-
Tb60 (PVY isolate control)	**267 bp**	**689 bp**	**532 bp**	**853 bp**	**-**
ME-173 (PVY isolate control)	**267 bp**	**689 bp**	**532 bp**	**853 bp**	**-**
N1 (PVY isolate control)	**181 bp**	**689 bp**	**441 bp**	**853 bp**	**-**
HR1(PVY isolate control)	**181 bp**	**452 bp**	**441 bp**	**633 bp**	**1307 bp**

[a)] bp, base pairs

[b)] Negative by RT-PCR

[c)] Nt, not tested

Table 5 Characterization of PVY^{NTN} and PVY^{EU-N} isolates Fab31, 37 and wild potato using RT_PCR with two sets of primers

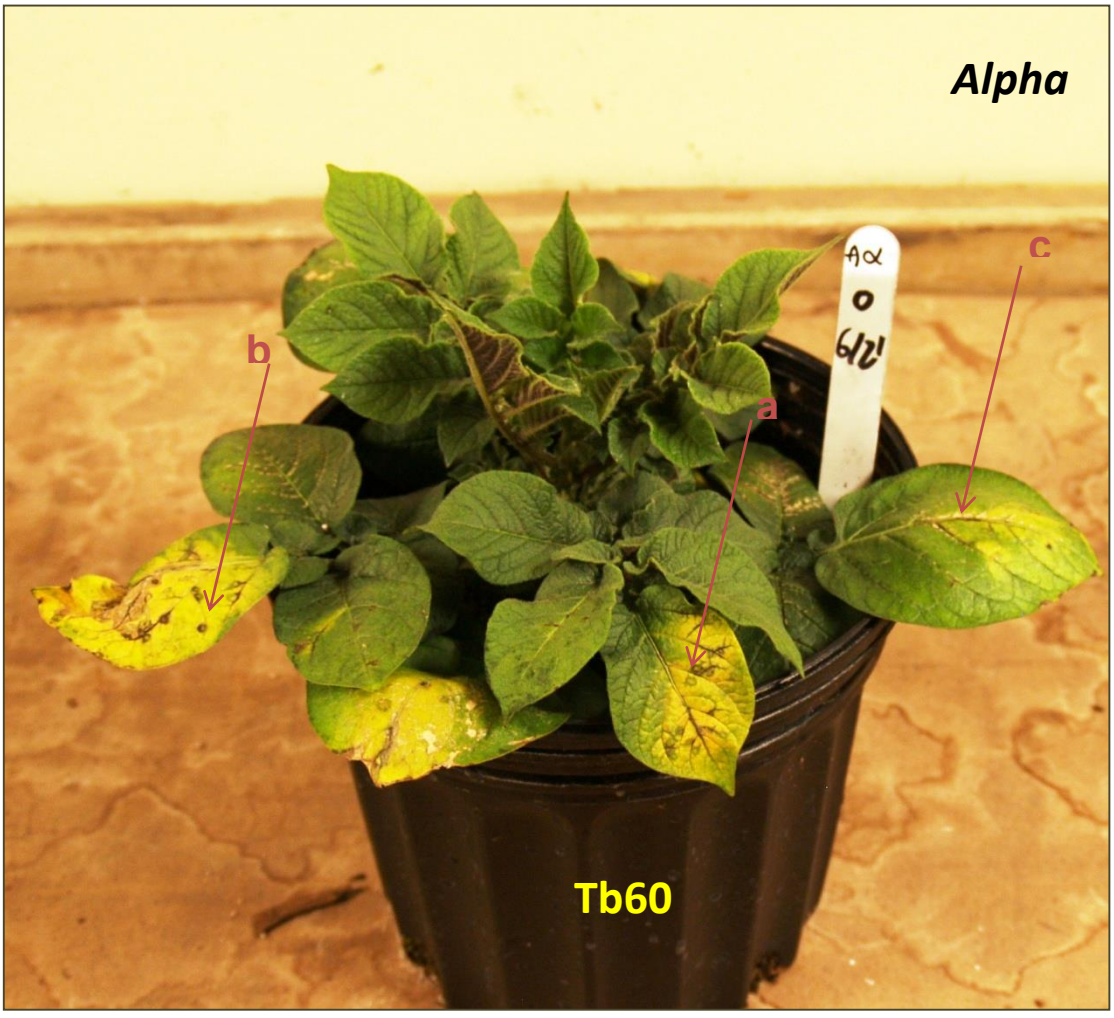
Characterization of field isolates Mex 31, Mex 37 and an isolate from wild potato Mex 43, using reverse transcriptase polymerase chain reaction with two sets of multiplex primers (Lorenzen *et al.*, 2006 and Chikh-Ali *et al*., 2010).

Primer set	**Lorenzen**	**Chikh-Ali**	**Strain identification**
Mex31 (unknown red potato cultivar)	**181, 452 bp** [a]	**441, 633, 1307 bp**	**NTN**
Mex37 (Fabula)	**181, 452 bp**	**441, 633, 1307 bp**	**NTN**
Mex43 (Wild Potato)	**328, 398 bp**	**398, 633, 1307 bp**	**Eu-N**
Tb60 (PVY isolate control)	**267, 689 bp**	**532, 853 bp**	**O**
ME-173 (PVY isolate control)	**267, 689 bp**	**532, 853 bp**	**O**
N1 (PVY isolate control)	**181, 689 bp**	**441, 853 bp**	**N-Wi**
HR1 (PVY isolate control)	**181, 452 bp**	**441, 633, 1307 bp**	**NTN**

[a)] bp, base pair

Figure 1 Typical symptoms elicited by the Tb60 isolate of PVY (strain PVY^{O}) in potato cultivar Alpha, 15 days post inoculation. These are necrotic rings, later developing into local necrotic lesions, which later coalesce and develop into vein necrosis with subsequent total necrosis of inoculated leaves

Symptoms of local HR visible on cultivar Alpha 15 days post-inoculation with isolate Tb60 (PVYO). These are green rings[a], local necrotic lesions[b], and vein necrosis on inoculated leafs[c].



PVY^{O} infection (Tb60): 15 dpi

Figure 2 Comparison of the symptoms in inoculated leaves of the potato cultivar Alpha after inoculation with PVY isolates Tb60 (strain PVY^{O}), M3 (strain PVY^{Z}-NTN), and Mont (strain PVY^{Eu-N}); (A) – 15 days post inoculation (dpi), and (B) – 21 dpi.

Different types of symptoms that developed on potato cultivar Alpha after mechanical inoculation with three isolates of PVY – M3 (PVY^{Z}-NTN), Tb60 (PVY^{O}), and Mont (PVY^{N}), (A) 15 days post inoculation (dpi), and (B) 21 dpi.

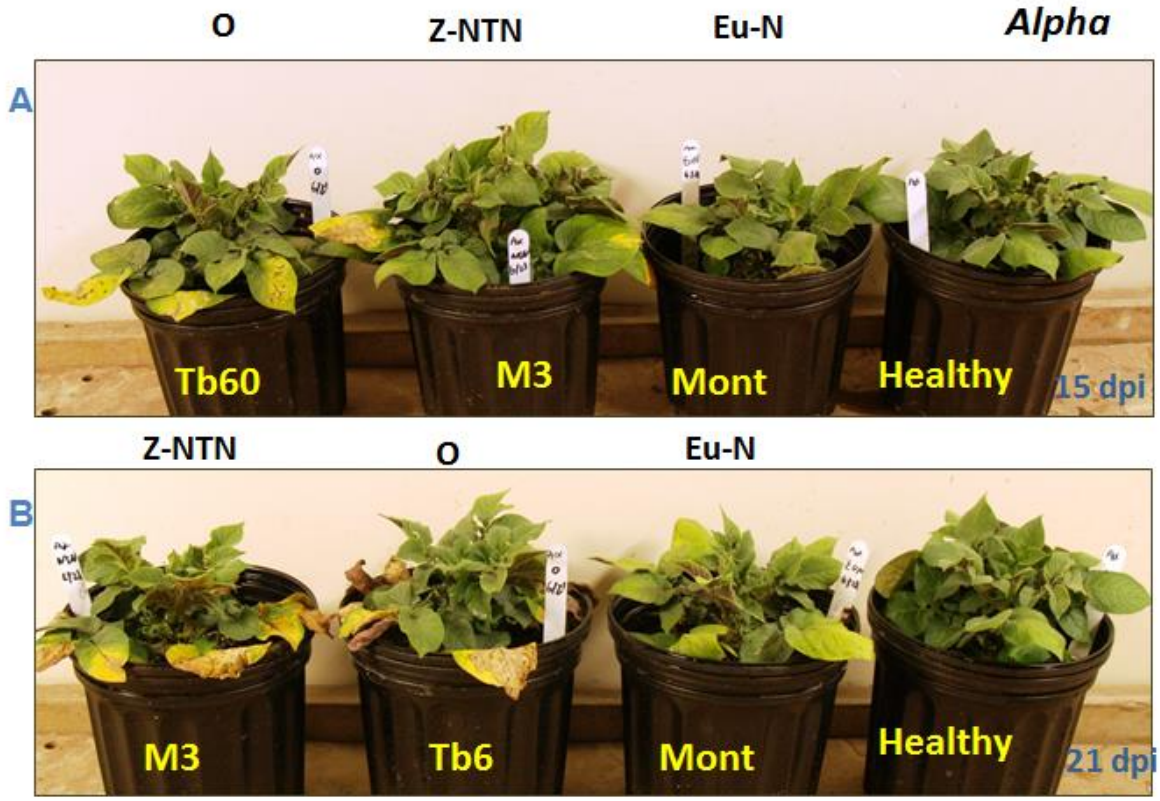


Figure 3. Reaction in local, inoculated leaves of potato cultivars Alpha and Mondial against PVY isolates M3 (strain Z-NTN) and Mont (strain Eu-N), 16 days post inoculation (pi). Red arrows indicate HR reaction on inoculated leaves.

Different symptoms after a successful infection on potato cultivars Alpha and Mondial after mechanical inoculation with two isolates of PVY – M3 (PVY^{Z}-NTN) and Mont (PVY^{N}), 16 days post inoculation.

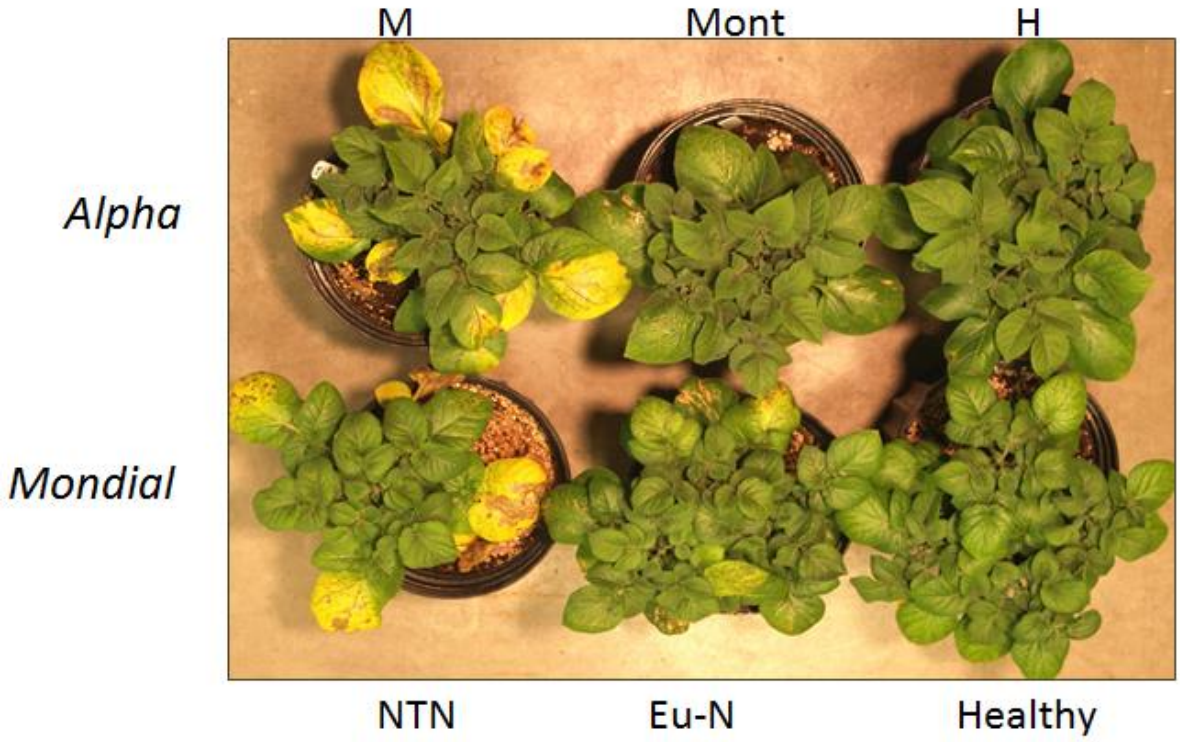


Figure 4.Phylogenetic tree of MSU_457 and MSU_470 isolate.

Phylogenetic analysis of full genome nucleotide sequences of *Potato virus Y* (PVY) Isolates of the genome of representative PVY isolates inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the nodes. Only bootstrap values higher than 50% are retained. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

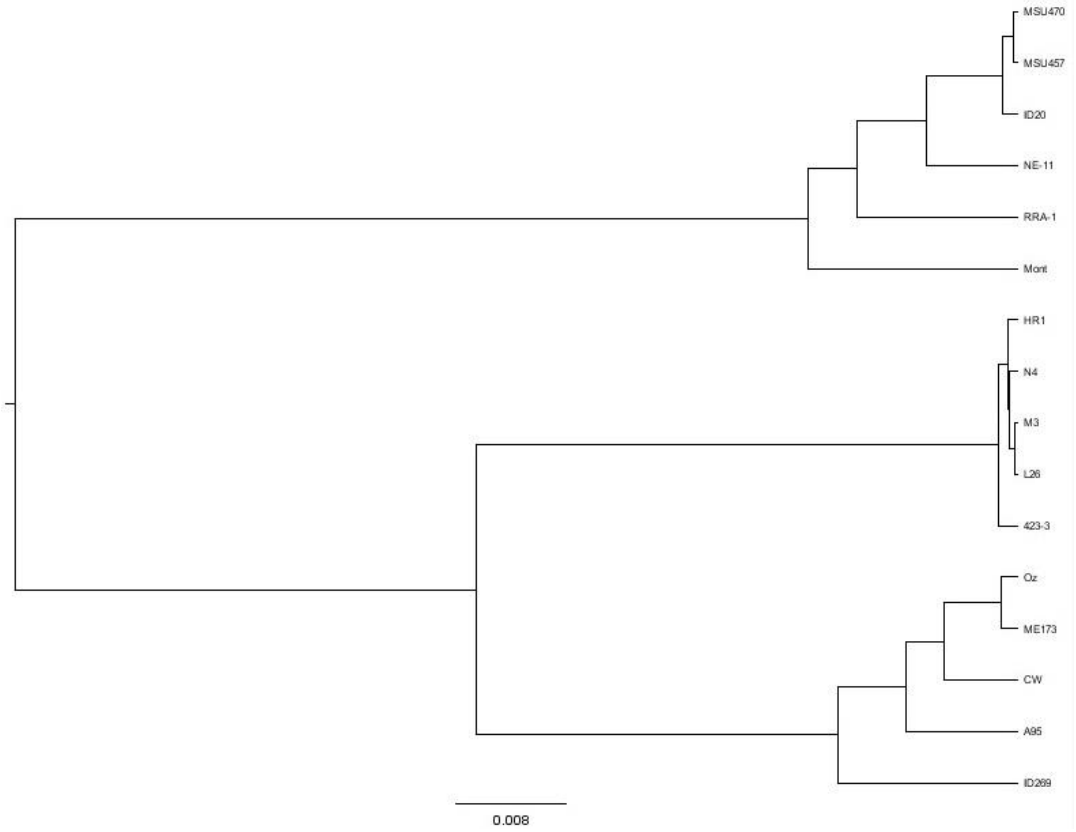


Figure 5.Distance plot analysis of the whole genome sequence of PE_17 against three control sequences, Oz (PVY^{O}), Mont (PVY^{N}), and ID20 (PVY^{NE11}).

Distance plot of the whole genome of isolate PE_17 (A) scanned against three controls, Oz (PVY^{O}), Mont (PVY^{N}), and ID20 (PVY^{NE11}) (B). The similarity pattern shows a recombinant structure of PE_17.

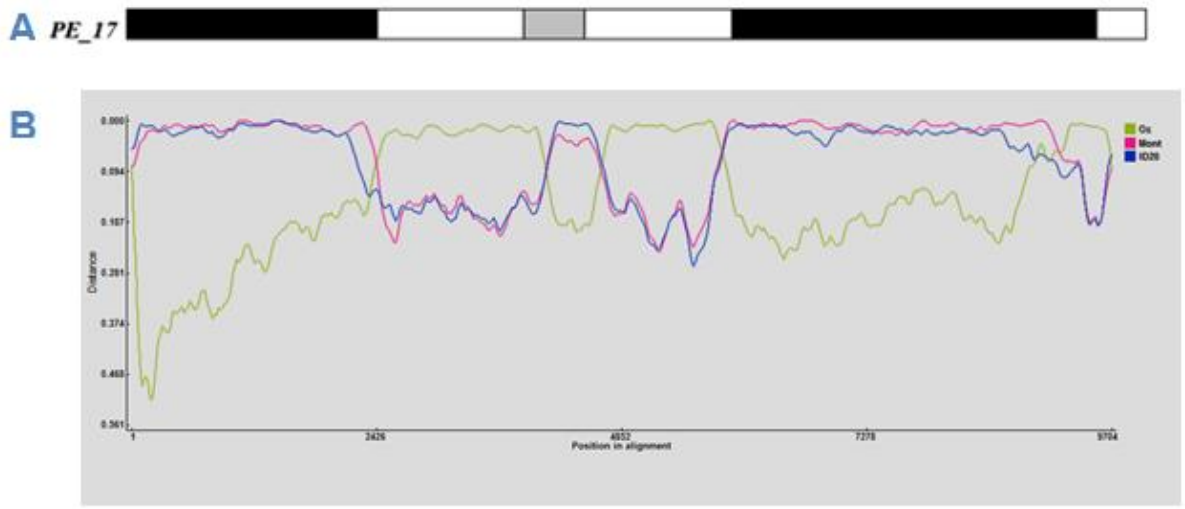


Figure 6. Comparison of three different PVY isolates PE-17, MSU-457 and MSU-470 with the parental strains PVY^{Na-N}, PVY^{O} and PVY^{EuN} and two other recombinant strains PVY^{NTN} and PVY^{NE-11}

Genomes of the three sequenced PVY isolates, PE_17, MSU457, and MSU470, in comparison with the parental, non-recombinant strains of PVY, and with two reference, recombinant strains, PVY^{NTN} and PVY^{NE11}. Shading with different colors represents homologous segments of the genome between different strains of PVY. Red arrows mark positions of the recombinant junction in the two types of the NE11 strain.

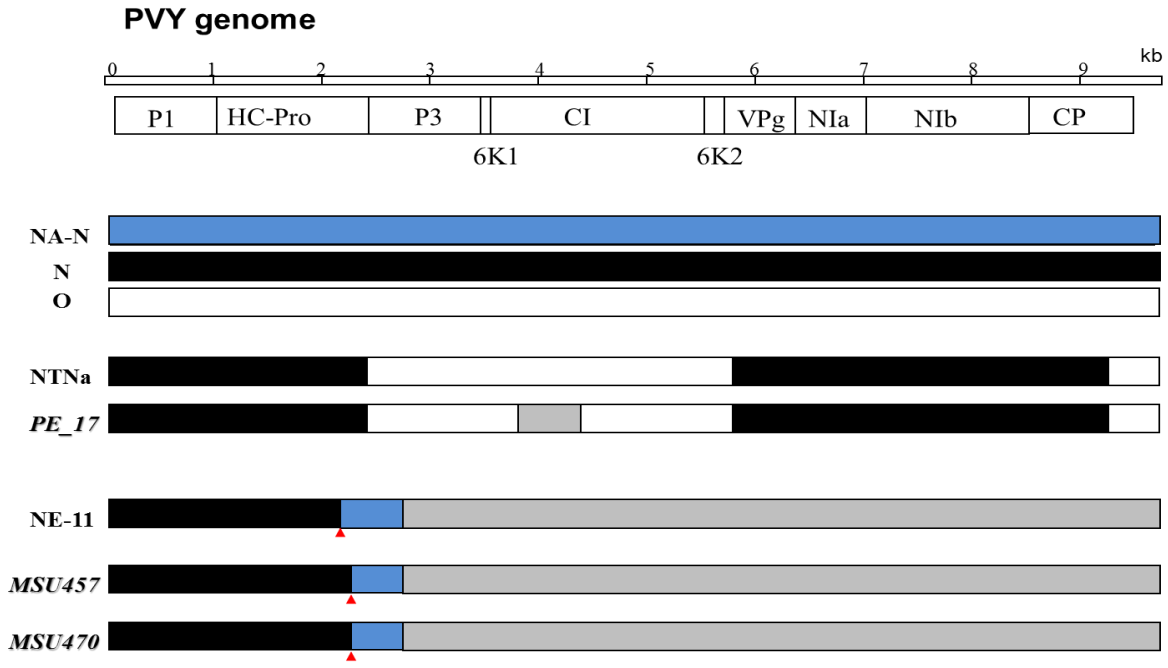


Figure 7. Phylogenetic tree of the whole genome sequences for several PVY^{N} sequences, including the Mex43 isolate (PVY^{N}) from a wild potato sample collected in Jalisco,

Phylogenetic tree for PVY^{N} isolates including the Mex43 whole genome, created using the Maximum Likelihood algorithm. The PVY$^{Na\text{-}N}$ isolate RRA-1 was used as an outgroup.