DEVELOPMENT OF A PEPPER (*Capsicum annuum* L.) HYBRID VARIETY WITH RESISTANCE TO POTATO VIRUS Y (PVY) USING MOLECULAR BREEDING

by

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Dissertation abstract

Pepper (*Capsicum annuum* L.) is an important vegetable crop grown and consumed worldwide. Potato virus Y (PVY) is a globally economically important pathogen which significantly reduces the yield and quality of cultivated pepper. The virus is considered as a major limiting factor to the economic production of pepper in the province of KwaZulu-Natal (KZN) in the Republic of South Africa (RSA). Many applied practices to control the spread of PVY are ineffective to mitigate the losses incurred by many farming communities across the KZN province. Therefore, the objectives of this study was to determine the full genome sequence of a PVY isolate from KZN, to identify resistance alleles in commercially available pepper varieties in KZN and to develop a pepper hybrid variety with resistance to PVY using a molecular breeding strategy

The first part of the study was conducted to determine the first full genome sequence of a PVY isolate (JVW-186) infecting pepper from KZN. The complete genome sequence of JVW-186 was assembled from overlapping RT-PCR clones using MEGA 5 software. Individual ORFs were identified using the nucleotide data base NCBI and aligned using CLUSTALW. RDP4 software was used to identify recombination junctions in the sequence alignment of JVW-186. CLC Main Workbench 6 software was used to determine the nucleotide sequence similarity of recombinant and non-recombinant fragments of JVW-186 in conjunction with ten PVY parental isolates. Based on sequence data, virus morphology and the coat protein size as determined by SDS-PAGE analysis, the identity of the isolate JVW-186 was confirmed as PVY. Phylogenetic trees were constructed from all recombinant and non-recombinant segments of the sequence by the maximum likelihood method using MEGA 5 software. The full length sequence of JVW-186 consisted of 9700bp. Two ORF's were identified at position 186 and 2915 of the sequence alignment encoding the viral polyprotein and the frameshift translated protein P3N-PIPO, respectively. RDP4 software confirmed two recombination breakpoints at position 343 and 9308 of the sequence resulting in four segments of the genome. At each recombination event, a 1021-bp fragment at the 5'

end in the region of the P1/HC-Pro protein and a 392-bp fragment in the region of the coat protein shared a high sequence similarity of 91.8 % and 98.89 % to the potato borne PVY^{C} parental isolate PRI-509 and the PVY^{O} parental isolate SASA-110 respectively. The non-recombinant fragment 1 clustered within the C clade of PVY isolates; however the large 7942-bp fragment 3 did not cluster within any of the clades although it shared > 80% nucleotide sequence similarity to other PVY isolates used in this study. Our results suggest that isolate JVW-186 is a novel recombinant strain of PVY that could have evolved due to the dynamics of selection.

The second part of the study aimed to evaluate different pepper lines for resistance to PVY. Two recessive alleles (*pvr2*^{1} and *pvr2*^{2}) located on the *pvr2-eIF4E* locus are known to confer resistance to the virus. To this end, six pepper lines were challenged with PVY infected *Nicotiana tabacum* cv. Xanthi leaf material using mechanical inoculation under greenhouse conditions. Each line was assessed for resistance to PVY by visual screening for disease severity and quantitative enzyme linked immunosorbent assay (ELISA) for virus load. Pepper lines were further characterized using tetra-primer ARMS-PCR (amplification refractory mutation system polymerase chain reaction) to identify and differentiate the presence of homozygous/heterozygous resistance alleles that confer PVY resistance. Evaluations revealed two resistant pepper lines (Double Up and Cecelia) and varying levels of susceptibility in the other four pepper lines challenged with PVY. The most susceptible pepper line was Benno, although high levels of susceptibility were observed in three other lines (IP, Mantenga and Excellence). The *pvr2*$^{+}$ allele was positively identified in all the susceptible pepper lines using the T200A tetra-primer which confirms that the presence of this allele is dominant for PVY susceptibility. Double Up and Cecelia were genotyped homozygous *pvr2*^{1}/*pvr2*^{1} and *pvr2*^{2}/*pvr2*^{2} respectively, and remained asymptomatic throughout the trial which indicates that these alleles confer resistance to the isolate of PVY used in this study. The information generated in this study can be incorporated into breeding programs intended to control PVY on pepper in KZN.

The final part of the study focused on the development of resistant varieties as the best alternative to manage PVY diseases on pepper. Homozygous F_2 pepper lines were

developed from local germplasm carrying PVY resistance genes (*pvr2*^{1} and *pvr2*^{2}) using marker assisted selection (MAS). The F_1 progeny was obtained by crossing a homozygous *pvr2*^{1} (resistant) 'Double Up' cultivar with a heterozygous susceptible (*pvr2*$^{+}$/*pvr2*^{2}) 'Benno' cultivar. F_1 and F_2 generations were assessed for the presence of PVY resistance/susceptibility alleles (*pvr2*$^{+}$/*pvr2*^{1}/*pvr2*^{2}) at the *pvr2-eIF4e* locus using the tetra primer amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) procedure. Negative selection was carried out using the tetra-primer T200A marker to detect the *pvr2*$^{+}$ (susceptible) allele. All F_1 progeny displaying the *pvr2*$^{+}$ allele were eliminated from further study. All 302 plants belonging to 29 F_2 families expressing homozygous recessive traits were tested via mechanical inoculation for their response to PVY infection and resistance to PVY was confirmed in all selected families based on symptomatology in greenhouse house screens using double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). These results show that ARMS-PCR can be used to successfully screen pepper genotypes for alleles that confer PVY resistance thereby contributing to the improvement of pepper production using molecular breeding approaches.

Declaration

I, **Vaneson Moodley**, declare that

i. The research reported in this thesis, except where otherwise indicated, is my original work.

ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other persons‟ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Dedication

To my parents for encouraging me to be a person of value.

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Introduction to thesis

The importance of pepper

Pepper is a widely cultivated vegetable and spice crop in many parts of the world (Bosland and Votava, 2000). The Republic of South Africa (RSA) has a substantial pepper production industry which may not rank with leading producers like China, Mexico and Indonesia; however, it generates revenue in excess of 290 million ZAR (Directorate of Agricultural statistics, 2012). The crop is used as an essential constituent in many globally distributed food industries (Bosland and Votava, 2000). Pepper is a member of the *Solanaceae* family originating from South and Central America where it was domesticated 7000 BC (Kumar *et al*., 2011). Characteristics associated with pungency and oleoresin (color) of pepper is used extensively as a spice in food products as well as in pharmacology (Kumar *et al*., 2011). In 2011, global pepper production reached 1.84 million ha with a total harvest of more than 29.6 million tonnes (FAO, 2011). China is by far the largest producer of pepper, contributing more than 50 % (15.2 million tonnes) of the total production of pepper throughout the world (FAO, 2011). In addition to their nutritional value, peppers are high value crops which can provide an excellent income-generating opportunity for small-scale/subsistence farming communities (Selleck and Opena, 1985). However, the economic production of pepper is under threat from a variety of pest and pathogens. While a great diversity of pests and pathogens have been documented over the years, viruses indisputably remain one of the most biologically intriguing groups of pathogens affecting modern day agriculture. The inability to cure virus infected plants and reduce preliminary infections through vector transmission without the need for using environmentally toxic pesticides warrants alternative indirect methods of control for viral diseases on plants.

Potato virus Y (PVY)

Potyviruses are the largest group of plant infecting RNA viruses comprising about one third of all known plant viruses (Caranta *et al*., 2011). Approximately 180 potyvirus species are capable of infecting 30 currently registered plant families (Caranta *et al*., 2011). Potato virus Y (PVY) is the type member of the potyvirus group which is

naturally transmitted by aphids in a non-persistent manner to several economically important crop species including pepper (*Capsicum annuum* L.), tomato (*Solanum lycopersicon* Mill.), potato (*Solanum tuberosum*) and tobacco (*Nicotiana* spp.) (De Bokx and Huttinga, 1981; Shukla *et al*., 1994). PVY was first reported by Smith (1931) from symptomatic potato crops during a field survey and has since been identified in agricultural communities throughout the world. The virus is now ranked as the fifth economically most important viral pathogen infecting agricultural crops (Scholthof *et al*, 2011). In the early 1980's PVY became common among farming communities in RSA (Thompson *et al*., 1987). In the KwaZulu-Natal (KZN) province, PVY was identified as the key constraint of pepper production (Budnick *et al*., 1996).

Protecting pepper crops from the threat of pathogens such as PVY is of great economic importance. Drawbacks associated with currently available strategies to manage PVY disease on pepper have created a need to develop alternative methods of control. Genetic studies of the host in relation to the infecting pathotype allow the opportunity to develop resistant varieties with durable natural resistance. PVY comprises a variety of strain groups and pathotypes that are genetically diverse. In pepper, pathotype-specific resistance to PVY is mediated by an allelic series of recessively inherited genes at the *pvr2-elF4E* locus (Rubio *et al*., 2008). A combination of molecular genetics and plant breeding techniques offer great potential to mitigate losses incurred by the KZN pepper industry as a result of PVY infection.

Marker-assisted selection (MAS)

Marker-assisted selection (MAS) is a valuable tool to identify genetic resources that can be incorporated into breeding programs. Additionally, these genetic markers can be used to assess parental lines for desired characteristics before crossing (Brumlop and Finckh, 2011). Prior to the advent of molecular markers in the late 1970's, plant breeders were dependent on phenotypic selection which relies on internal and external traits such as disease resistance, yield, or quality traits (Brumlop and Finckh, 2011). The use of phenotypic selection to achieve desired/improved germplasm is a long process and can easily exceed 10 years (Brumlop and Finckh, 2011). According to Xu and Crouch (2008), the greatest benefits of genetic markers in molecular breeding

programs are the possibilities of making breeding progress in a shorter period of time as compared to conventional breeding, as well as to accurately assemble desired traits with minimal unintentional losses.

Foolad and Sharma (2005) defined MAS as the use of associated markers to select for traits based genotype rather than phenotype. The introduction of MAS in the latter part of the 19th century spurred a great deal of interest among plant breeders with the hope of reducing the number of breeding cycles usually required for conventional plant breeding (Mazur, 1995). Furthermore, allelic variation of agronomically important genes can be distinguished using this tool (Peleman and Van Der Voort, 2003).

DNA polymorphisms that account for phenotypic characteristics of interest are used for the development of highly efficient molecular markers which provide an optimal tool to track the gene of interest in breeding progeny (Anderson and Lubberstedt, 2003). Additionally, these markers are particularly important for distinguishing an individual with resistance specificities against pathogens and their strains (Rubio *et al*., 2008). Pepper has the largest allelic series at the *pvr2-elF4E* locus in-which mutations regulate strain specific resistance to PVY and other viruses (Charron *et al*., 2008).

Rubio *et al* (2008), designed a set of functional markers based on single nucleotide polymorphisms at *pvr2-elF4E* resistance locus in pepper that confer resistance to PVY using the tetra primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR) procedure. This technique is able to differentiate both homozygous and heterozygous genotypes using a set of four primers in a single polymerase chain reaction (PCR) run (Rubio *et al*., 2008). Four recessive alleles i.e. *pvr2*$^{+}$, *pvr2*^{1}, *pvr2*^{2} and *pvr2*^{3} that control pathotype mediated resistance against PVY can be distinguished at the *pvr2-elF4E* locus using the tetra-primer ARMS-PCR procedure designed from single nucleotide polymorphisms (SNP) (Rubio *et al*., 2008). The use of these allele specific co-dominant markers can be used to screen large populations of breeding progeny at the *pvr2* resistance locus in pepper (Rubio *et al*., 2008).

The constraints associated with current efforts to curb the spread of PVY require an alternative approach. Tetra-primer ARMS-PCR is a low cost genetic tool which can be used to successfully screen pepper genotypes for alleles that confer PVY resistance thereby contributing to the improvement of pepper production. Moreover, the use of MAS can substantially lessen the effort required for conventional breeding.

Research Objectives

The objectives of this study were therefore to:

1. Characterize the predominant PVY isolate infecting pepper in KZN using whole genome sequencing and phylogenetic analysis.
2. Evaluate pepper lines for resistance against the infecting isolate of PVY in KZN.
3. Develop homozygous pepper hybrids with resistance to PVY using molecular markers.

Dissertation Structure

This dissertation comprises five chapters. Chapter 1 is a review of literature which outlines the importance of pepper; the implications associated with PVY infection of pepper; characteristics, distribution and spread of PVY; strategies to identify and mitigate losses associated with PVY infection of pepper. Chapter 2 focuses on the molecular characterization and whole genome sequencing of an isolate PVY infecting pepper in KZN. Chapter 3 deals with the screening of pepper lines to an isolate of PVY infecting pepper in KZN. Chapter 4 addresses the development of homozygous pepper varieties with resistance to the infecting pathotype of PVY in KZN. Chapter 5 provides an overview of the major findings of the research and identifies information gaps followed-by suggestions on future research to fill these gaps

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Chapter 1

Literature Review

1.1 General Introduction

The dietary benefits of vegetables in human nutrition provide a source of essential vitamins and minerals that complement starchy staple foods (Nono-Womdin, 2003). Pepper (*Capsicum annuum* L.) is one of the most widely cultivated vegetable crops throughout the world that are an important source of antioxidant and anti-inflammatory phytonutrients (Bosland and Votava, 2000). Although widely grown and consumed, the yield of vegetables in Africa remains low in comparison to other parts of the world, which can be attributed to various biotic and abiotic factors among which viral diseases impose significant production constraints and are challenging to control (Nono-Womdin, 2003).

Plant viruses are a major limiting factor to the economic production of crops on a worldwide basis. Although viruses are relatively simple genetic entities, the mechanisms fundamentally employed during infection, in addition to the manner by which plants resist these effects are not well understood (Kang, 2005a). The detection and identification of viruses are important aspects of studying viruses. Accurate virus detection and identification is crucial if sustainable control/management strategies of diseases caused by these viruses are to be implemented.

The objectives of this literature review were to

I. Provide background information of the importance and distribution of pepper in KwaZulu-Natal (KZN), Republic of South Africa (RSA) and abroad.
II. Provide current information relating to the losses incurred by pepper industries as a result of PVY infection.
III. Review current strategies to detect and manage PVY disease on pepper.

1.2 The Pepper Crop

Bell peppers are members of the *Solanaceae* (nightshade) family along with tomato (*Solanum lycopersicum* Mill.), potato (*Solanum tuberosum*), tobacco (*Nicotiana* spp.), eggplant (*Solanum melogena*), cayenne pepper (*Capsicum annuum*) and chili pepper (*Capsicum chinense*). The *C. annuum* complex which comprises two other closely related species i.e. *C. chinense* and *C. frutescens* are presently the most widely cultivated anywhere in the world (Pickersgill 1997).

1.2.1 Physiology

The species name *annuum* means annual, however, peppers can develop into large densely branched woody perennial shrubs (up to 75 cm in height) over several growing seasons in the absence of winter frost (Courteau, 2012). The white bell-shaped flowers (often with 5 lobes and contain 5 bluish stamens) are borne single in the axils (Courteau, 2012). The leaves are simple and alternate with smooth entire margins and the fruit are blocky or elongated juiceless berries that contain many seeds and may ripen to green, yellow, orange, red or purple.

1.2.2 Cultivation

Capsicum. annuum grows optimally in warm climates with temperatures ranging between 20 - 25°C (Anonymous, 2013) and is particularly vulnerable to frost damage (Bosland and Votava, 1999). Slow seed germination is generally associated with low soil temperature; however seedling emergence accelerates at temperatures of 24 - 30°C (Bosland and Votava, 1999). Bell pepper has a weekly water requirement of 25 mm for the first five weeks followed by 35 mm thereafter; an excessive supply of water can have adverse effects on the development of flowers and fruit leading to fruit rot (Coertze and Kistner, 1994). The fertilizer requirement for bell pepper production is dependent upon factors pertaining to the type of soil, soil nutrient status and the pH (Anonymous, 2013); therefore an assessment of the soil is generally required prior to planting (Coertze and Kistner, 1994). According to the FSSA (2007) the fertilizer quantity for bell pepper is 1.5 – 3 kg N, 0.2 - 0.4 kg P and 2 - 4 kg K per tonne of fruit harvested.

1.2.3 Origin and distribution

The genus *Capsicum* which originated in the American tropics (domesticated in the Mexican highlands) is currently grown throughout the American, African and Asian tropics, where their fruits are particularly valued for the exotic flavour they add to the local diet (Pickersgill, 1997). In RSA the major pepper production areas (field and tunnels) include Gauteng (highveld and lowveld), Northern Cape, Eastern Cape, Western Cape, Limpopo and KZN (Anonymous, 2013).

1.2.4 Importance of pepper in KwaZulu-Natal

These crops are grown extensively in RSA mainly for fresh produce. The province of KZN has a substantial pepper industry and the cultivation of this crop is favored by the general climatic conditions of the province. Consequently, the detection and identification of viruses such as potato virus Y (PVY) and their effect on these solanaceous crops are crucially important to plant pathologists globally.

1.2.5 Potato virus Y (PVY) infecting pepper

PVY was first reported in potato (*Solanum tuberosum*) in 1931, together with a group of pathogens associated with potato degeneration, a disorder recognized since the 18th century (Smith, 1931). The first described PVY isolate, in the early 1930s, was assigned to the non-necrotic PVY^{O} group which has remained predominant among PVY isolates gathered during field surveys (Smith, 1931). Conversely, recent studies undertaken in Europe and North America have demonstrated that the balance between necrotic and non-necrotic PVY isolates has since changed (Piche *et al.*, 2004; Lindner and Billenkamp, 2005). It seems that a higher incidence of necrotic isolates is now present in natural PVY populations (Rolland *et al*., 2009). PVY is one of the five most economically destructive plant viruses affecting field grown solanaceous crops worldwide (Milne, 1988; Shukla *et al*., 1994).

Budnik *et al* (1996) identified PVY as the most devastating viral disease infecting pepper in KZN. Follow-up studies confirmed similar results for tomatoes, potatoes and pepper grown in KZN (Ibaba and Gubba, 2011). Although PVY is not currently an epidemic in RSA or KZN it is does substantially reduce the yield of cultivated pepper infected fields (Ibaba and Gubba, 2011). Currently available methods to mitigate the

incidence of PVY have been met with limited success; hence the development of resistant varieties is the best alternative to manage PVY on pepper. By screening various cultivars of pepper for resistance to PVY, genotypes expressing high levels of resistance can be incorporated into breeding programs that may confer durable natural and safe methods of resistance.

1.3. Classification of PVY

PVY, the type member of the genus *Potyvirus*, family Potyviridae, is one of the most economically important pathogens infecting several solanaceous species including pepper (*Capsicum* spp.), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum* Mill.) crops, to which it is non-persistently transmitted by several aphid species (De Bokx and Huttinga, 1981; Shukla *et al.*, 1994).

1.4. PVY Particle Morphology

PVY virions are non-enveloped, filamentous, flexuous rods (Figure 1.1) with a modal length of 740 nm and a width of 11 nm (Shukla *et al.,* 1994). Patterns in relation to the optical diffraction indicate a helical (mean pitch of the helix being 3.3 nm) structure of the virions (Verma *et al*., 1968; Goodman *et al*., 1976) with an approximate number of 7.7 protein subunits per turn of the helix (Veerisetty, 1978, Tollin and Wilson, 1988).

1.5. Genome Organization

The monopartite PVY genome comprises a single-stranded, positive-sense RNA molecule about 10 kb in length (Shukla *et al.*, 1994) which consists of approximately 2000 copies of coat protein (CP) that forms a cylindrical inclusion body (CIb) which is reflected as a key phenotypic criterion for distinguishing a potyvirus infection from other virus groups (Talbot, 2004). The 10 kb positive sense viral RNA which is encapsidated by the CIb has a non-translated 5'-terminal region (5'-NTR) together with a 3'-poly-A tail and contains a single extended open reading frame which acts directly as mRNA

(Dougherty and Carrington, 1988; Van der Vlugt *et al.,* 1989). The 5'-NTR comprises 144 nucleotides that has a considerably higher adenine to guanine ratio, and is associated with a viral genome linked protein (VPg) (Carrington and Freed, 1990). The VPg which functions to enhance transcription is also a unique virulence determinant toward overcoming resistance genes in pepper (Mourey *et al*., 2004).

The 5'-leader sequence possesses an internal ribosome entry site (IRES) that allows translation of RNAs in a cap-independent manner by means of a mechanism similarly employed by eukaryotes and cap-independent translation regulatory elements (CIREs) (Basso *et al.,* 1994). The viral RNA encodes a large single (350 kDa) polypeptide which is cleaved by three viral proteases (NIa, HC-Pro and P1) which undergoes co-translational and post-translational cleavage yielding nine functional proteins (Basso *et al.,* 1994). These proteins include, P1 (P1 Protein), HC-Pro (Helper Component Proteinase), P3 (P3 Protein), 6K1 (6 kDa Protein 1), CIb (Cylindrical Inclusion body), 6K2 (6 kDa Protein 2), VPg (Viral Genome-linked Protein), NIa-Pro (Nuclear Inclusion Protein a, Proteinase domain), NIb (Nuclear Inclusion Protein b) and the CP (Coat Protein) (Talbot, 2004; Fig 1.2). A recent study has reported the presence of a second short open reading frame (Chung *et al.*, 2008) embedded within the previously described large open reading frame.

1.6 Protein Function

The size and function of each protein have been summarized according to (Urcuqui-Inchima *et al.*, 2001; Fig 1.1). P1, a trypsin-like serine proteinase (34-64 kDa) plays a role in symptomology and C terminal autocleavage. The helper component protein (HC-Pro; 50 kDa) is a multifunctional protein involved in aphid transmission, local/systemic movement, and synergism. The HC-Pro also functions as a suppressor of gene silencing. The P3 (37 kDa) protein is involved in plant pathogenicity and viral replication. The function of the 6K1 (6 kDa) protein remains unknown, however, the 6K2 (6 kDa) protein attaches the viral replication complex to the endoplasmic reticulum-like membranes of the host. CI, a 70 kDa RNA helicase and ATPase protein has been implicated in local movement of virus. The NIa (49 kDa) proteinase processes the

polyprotein in *cis* and *trans* to produce functional proteins also involved in genome replication (VPg; 25 kDa) and protein-protein interaction. The Nib (58 kDa) protein is an RNA-dependent RNA polymerase involved in genome replication and finally, the CP (coat protein; 30 kDa) which is multifunctional protein involved in virus assembly, aphid transmission and local and systemic movement (Urcuqui-Inchima *et al.*, 2001).

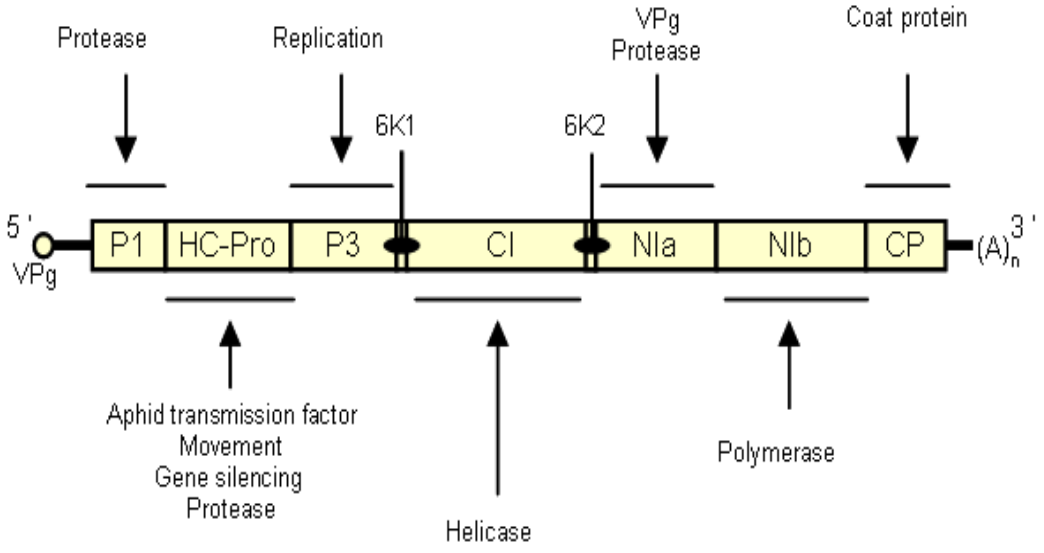

Figure 1.1 PVY genome characterization (Delaunay, 2007).

1.7 PVY Strains

Isolates of PVY have been classified based on their ability to induce vein necrosis (PVY^N) or not to induce vein necrosis (PVY^O) on *Nicotiana tabacum* cv. Xanthi leaves (Rolland *et al.,* 2009). Subsequently, the description of these two main PVY groups has enabled the development of numerous biological (Jones, 1990; Valkonen, 1997), serological (Rose and Hubbard, 1986; Cerovska, 1998; Ounouna *et al.*, 2002) and molecular (Weidemann and Maiss, 1996; Glais *et al.*, 2005; Kogovsek *et al.*, 2008; Rolland *et al.*, 2008) diagnostic tools capable of detecting and describing the diversity of PVY isolates. These tools have been characteristically designed based on specificity,

sensitivity and accuracy to differentiate, describe and assess PVY isolates in natural populations (Crosslin *et al.*, 2006).

PVY strains comprise a wide variety of genetically diverse isolates (Singh *et al*., 2008). These naturally occurring strain groups have been classified as PVY^{O} (common or ordinary strain), PVY^{N} (tobacco veinal necrosis strain) and PVY^{C} (stipple streak strain) according to serology, molecular assays and symptomology (Karasev *et al*., 2010). Extensive studies to determine the genetic diversity of potyviruses have been predominantly based on the coat protein (CP) gene (Shukla *et al*., 1994). An estimated 8% variation has been established along the entire 9.7kb PVY genome following complete genome sequences of numerous PVY strains belonging to the PVY^{O} and PVY^{N} groups (Karasev *et al*., 2011). More-over, a multitude of recombinants possessing spliced fragments of PVY^{O} and PVY^{N} sequences in their genomes have been identified in addition to these main parental genomes (Karasev *et al*., 2011).

Characteristic symptoms associated with PVY^{O} and PVY^{C} include mosaic, mottling and rugosity, whereas PVY^{N}, $PVY^{N:O}$ and PVY^{NTN} are defined by veinal necrosis (Karasev *et al*., 2010). PVY^{O} and PVY^{C} elicit similar symptoms on tobacco but differ in their ability to induce hypersensitive reactions (HR) in potato cultivars bearing the Ny_{tbr} (PVY^{O}) and *Nc* (PVY^{C}) resistance genes (Blanco-Urgoiti *et al*., 1998; Karasev *et al*., 2011). PVY^{N} and recombinant strains $PVY^{N}W$, $PVY^{N:O}$ and PVY^{NTN} (termed necrotic isolates) are separated from PVY^{O} and PVY^{C} in their ability to induce severe veinal necrosis in tobacco (Karasev *et al*., 2011). PVY^{C} has also been described as non-aphid transmissible and can be divided into two sub-groups denoted C1 and C2 (Blanco-Urgoiti *et al*., 1998). Isolates belonging to the C1 group are termed non-potato isolates which are able to infect both pepper and potato; the C2 group consists of potato infecting isolates that infect pepper with reduced efficiency (via aphid transmission) but not mechanically (Blanco-Urgoiti *et al.*, 1998; Romero *et al.*, 2001). The incidence of PVY^{C} has been reported in Europe, Australia/New Zealand, the Americas, and RSA (De Bokx and Huttinga, 1981; Brunt, 2001).

Tuber necrosis is a common symptom induced by PVY^{NTN} isolates in susceptible potato cultivars (Karasev *et al*., 2010). Although reports of these necrotic strains infecting

cultivated potatoes are fairly recent (1990s), they were present in North America as far back as 1960 (Kahn and Monroe, 1963; McDonald and Kristjansson 1993). These necrotic isolates have since become widely distributed. $PVY^{N}W$, $PVY^{N:O}$ and PVY^{NTN} are recombinant isolates that contain PVY^{O} and PVY^{N} segments within their genome (Revers *et al*., 1996; McDonald and Singh, 1997; Glais *et al*., 1998; Boonham *et al*., 1999; Singh *et al*., 2003).

Reports of numerous recombinant and non-recombinant isolates of PVY have been described from many parts of the world including RSA (Kerlan, 2006, Ibaba and Gubba, 2011). PVY^{O} and PVY^{N} are the most commonly identified strains in RSA (Thompson, 1997). The level of damage to crops infected with PVY is collectively determined by the stage of plant growth during which infection occurs, the type of infecting strain, the degree of resistance/susceptibility of the plant toward the infecting pathotype and the viral load (Warren *et al*., 2005).

The presence of recombinant breakpoints present in PVY^{NTN} and $PVY^{N}W$ isolates has been demonstrated (Fig. 1.2). According to studies conducted by Glais *et al* (2002), all $PVY^{N}W$ isolates have been shown to display one or two recombination breakpoints (P1/HC-Pro and HC-Pro/P3). PVY^{NTN} isolates (most of which cause potato tuber necrotic ringspot disease [PTNRD]) frequently display recombination breakpoints at the C-terminus of the coat protein gene and two other genomic regions (HC-Pro/P3 and CI/6K2) (Revers *et al*., 1996; Glais *et al*., 1998; Boonham *et al*., 1999). Interestingly, numerous tuber necrosis-inducing isolates have been found to possess a PVY^{N}-type genome, void of any recombination break points (Ohshima *et al*., 2000; Glais *et al*., 2001; Boonham *et al*., 2002a).

Pathotypes of PVY that infect pepper have been classified; O, 1 and 1-2 based on their ability to overcome the recessive resistance alleles ($pvr2^{+}$, $prv2^{1}$ and $pvr2^{2}$) at the *prv2* locus (Gebre-Selassie *et al.*, 1985). Furthermore, ‘common’ and ‘necrotic isolates within these pathotypes have been defined (d'Aquino *et al.*, 1995). Serological studies indicate that isolates of PVY infecting pepper are closely related to the PVY^{O} strain group; however no relationship has been established between serotypes and pathotypes (Soto *et al*., 1994).

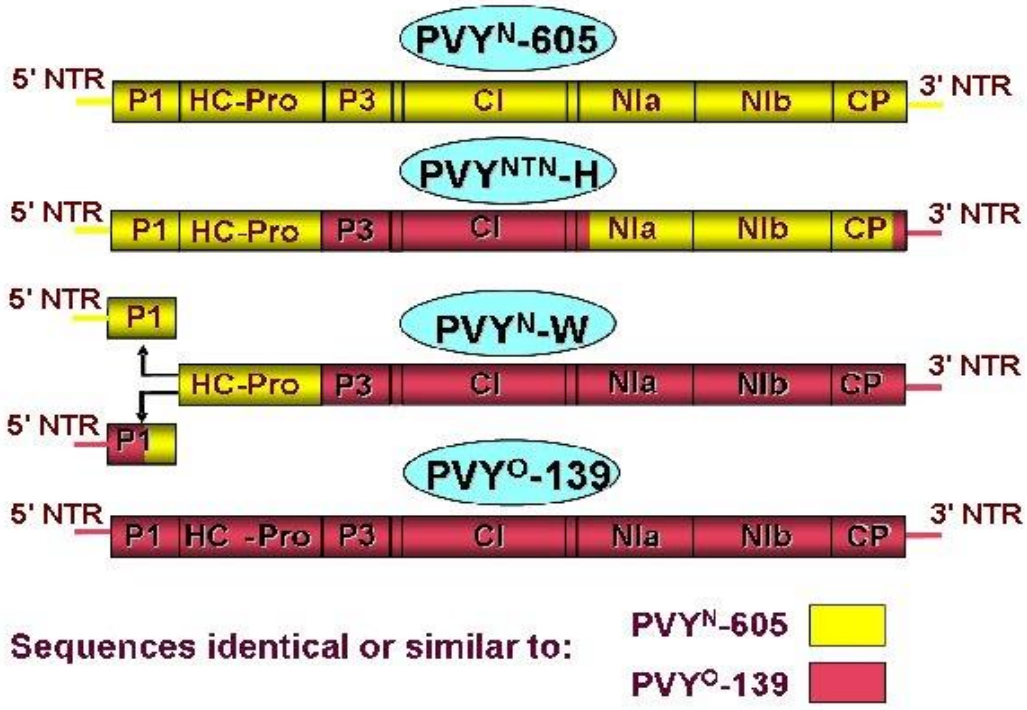

Figure 1.2 Genome organization of recombinant (PVY^{NTN} and PVY^{N}W) and non-recombinant (PVY^{N} and PVY^{O}) isolates of PVY (Glais *et al*., 2002).

While molecular studies of the coat protein amino acid sequences have proven useful in the taxonomy of potyviruses, it was unable to differentiate pathotypes of PVY infecting pepper (Llave *et al*., 1999). Although strains infecting pepper display a strong level of host specificity (Gebre-Selassie *et al.*, 1985; McDonald and Kristjanson, 1993; d'Aquino *et al.*, 1995), numerous isolates from potato and tobacco have been reported to infect pepper and tomato (McDonald and Kristjanson, 1993; Stobbs *et al*., 1994; Legnani *et al.,*1995). According to Kerlan (2006), the vast majority of PVY isolates are capable of infecting tobacco and tomato; however potato and pepper are more pathotype specific.

1.8 Geographical Distribution

PVY has a world-wide distribution wherever its natural hosts can be found. Field grown solanaceous crops including pepper, tomato, tobacco and potato cultivated in subtropical regions may have a higher incidence of the disease. PVY^{O} is reported to infect potato crops throughout the world (Jeffries, 1998). Although PVY^{N} occurs

extensively in Europe and South America, it is known to occur in Africa, Asia, New Zealand, Canada and to a lesser extent in North America (Weidemann, 1988; Fletcher, 1989; Ellis *et al*., 1997). PVY^{C} has been reported in RSA, Australia, India Europe, New Zealand and Ecuador (Ellis *et al*., 1997). PVY^{NTN} is common to most potato cultivation regions in the world (McDonald and Singh, 1996; Ohshima *et al*., 2000; Crosslin *et al*., 2002; Nie and Singh, 2003). PVY^{N}-W is widely established in Poland and emergent in other countries (Blanco-Urgoiti *et al*., 1998; Kerlan *et al*., 1999) whereas $PVY^{N:O}$ has been rife in Canada and the United States of America (Singh *et al*., 2003).

1.9 Host Range

PVY boasts a wide host range which comprises approximately nine families and includes major crops such as pepper (*Capsicum* spp.), tobacco (*Nicotiana* spp.), tomato (*Solanum esculentum*) and potato (*Solanum tuberosum*) (Jeffries, 1998). Moreover, ornamentals such as *Dahlia* and *Petunia* spp. together with an array of weeds including *Datura* spp., *Physalis* spp., *Solanum dulcamara*, *Solanum nigrum*, *Cotula australis* and *Capsella bursa-pastoris*are natural hosts of PVY (Jeffries, 1998; Fletcher, 2001). Edwardson and Christie (1997) report an experimental host range which identifies 495 species in 72 genera of 31 families. These include more than 287 species in 14 genera of the *Solanaceae*, 11 species of *Compositae*, 20 species of *Chenopodiaceae*, 25 species of *Leguminosae*, and 28 species of *Amaranthaceae*.

1.10 Transmission

PVY is mechanically transmitted through grafting and plant sap inoculation, or vectored by aphid transmission (Fig 1.3). PVY infected plant material in the field is often the result of alate aphids (Nie *et al*., 2011), and although these aphids cause substantial damage to crops, the greatest economic impact stems from their role as viral vectors (Radcliffe and Ragsdale, 2002; Halbert *et al.,* 2003). The moderate climate in RSA is conducive to asexual reproduction of aphids on indigenous and garden plants, which suggest that aphids are present throughout the year (Radcliffe and Ragsdale, 2002).

The green peach aphid (*Myzus persicae*) (Fig. 1.4), is the most efficient PVY vector, however, *Aphis fabae*, *Aphis gossypii*, *Aphis nasturtii*, *Macrosiphum euphorbiae*, *Myzus (Nectarosiphon) certus*, *Myzus (Phorodon) humuli* and *Rhopalosiphum insertum* pose a similar threat to the agriculture industry in their role as viral vectors (Halbert *et al.,* 2003; Warren *et al.,* 2005). Approximately 25 aphid species have been identified by the Agricultural Research Council-Vegetable and Ornamental Plant Institute (ARC-VOPI) of RSA which are able to effectively transmit PVY disease (Thompson, 1997). Aphids transmit the disease in a non-persistent, non-circulative manner which indicates a less intimate virus-vector interaction in comparison to circulative types (Gray, 1996).

Uzest *et al* (2010) identified the acrostyle which is a protein structure that occurs at the tip of the aphid stylets. The HC/Pro-virion complex attaches to the acrostyle allowing the transmission of virions to a susceptible host. Interestingly, the degree of PVY infection by aphids is attributed to host-plant selection and probing behavior, since virions are released from the stylets during salivation (Nie *et al*., 2011). The aphids acquire the virus from diseased plants and inoculate them onto suitable hosts by probing briefly into the epidermal tissue (Powell *et al.,* 2006).

The virus particles that attach to the acrostyle (acquired within a few seconds of feeding) can remain infectious for four to seventeen hours which limits the transmission of virions to shorter distances (Kostiw, 1975). Although these virus particles are only infectious for short periods of time, the frequency of virus acquisition and transmission within a field remains exceedingly efficient (Robert *et al.,* 2000).

Migrating aphids settle upon plants and arable weeds (typically biennial and perennial) in search of a suitable host to feed and reproduce (Kaliciak and Syller, 2009). Some of these non-crops and weeds may become infected with PVY and in doing so, function as a prime source of infection or natural virus reservoir for vectors to acquire and transmit to healthy crops (Kaliciak and Syller, 2009).

Reverse transcription polymerase chain reaction (RT-PCR) can be successfully applied to assess for the presence of PVY from viruliferous aphids caught in yellow pan traps containing propylene glycol (Nie *et al*., 2011). Viruliferous aphids trapped in propylene

glycol yield high quality RNA due to the preservative properties of this compound (Rubink *et al*., 2003; Vink *et al*., 2005; Nie *et al*., 2011). Consequently, a wide range of aphid species able to vector PVY in the field can now be identified using this technique.

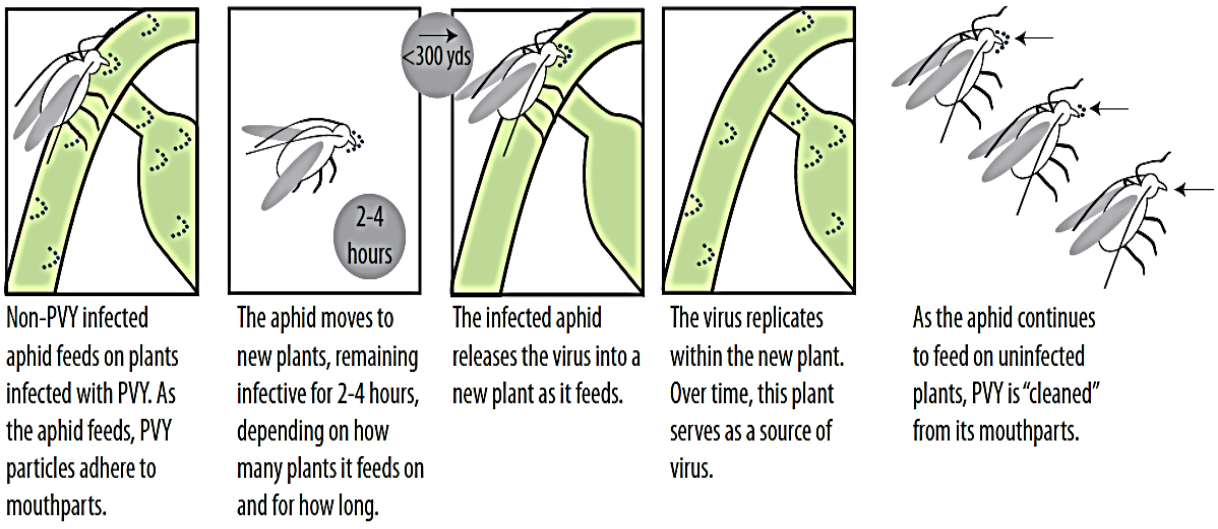

Figure 1.3 Vectored transmission of PVY (Schramm *et al*., 2011).

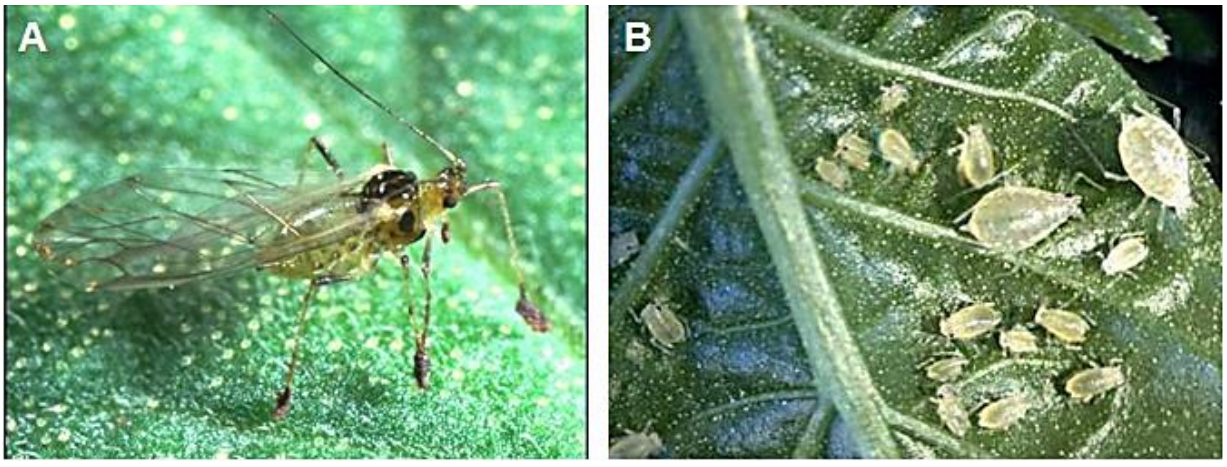

Figure 1.4 *Myzus persicae* (green peach aphid), the most effective aphid species of PVY infection. A: Winged adult (Kerlan, 2006); B: Wingless aphids on the underside of a pepper leaf. Adult females have 1-2 mm oval shaped bodies (Gammelgaard, 2011).

1.11 Symptoms on Pepper

Characteristic symptoms of pepper infected with PVY include systemic vein-clearing, accompanied by vein banding (mosaic along veins of leaves) and mosaic or mottling (Gebre Selassie *et al*., 1985; Cerkauskas, 2004; Fig. 1.5). Stunted growth followed by a reduced fruit set and size is commonly observed in pepper crops infected at an early growth stage. Symptom severity and the ability to set fruit are strongly influenced by the strain of PVY and the pepper cultivar (Belletti and Quagliotti, 1996).

Other symptoms include necrosis of the veins, petiole and stem leading to defoliation, loss of the apical bud and eventually death of the plant (Cerkauskas, 2004). The fruit of PVY infected pepper crops may be smaller and distorted with a mosaic making it unmarketable (Cerkauskas, 2004). PVY can infect crops at any stage of growth and often occurs in mixed infections which can enhance symptom expression in susceptible hosts (Cerkauskas, 2004). Furthermore, symptom severity increases in colder temperatures (Belletti and Quagliotti, 1996)

1.12 Detection of PVY

1.12.1 Enzyme linked immunosorbent assay (ELISA)

The accurate detection of casual agents of disease is essential to minimize losses incurred by the global agricultural industry. Various methods to detect plant viruses have been developed, however the specificity and rapid identification of viruses using serology is often favored (Clark and Adams, 1976). The use of various enzyme immunoassays to assess for the presence of pathogenic organisms is frequently adopted (Feldman *et al*., 1976). In the past enzyme-labeled antibodies were used exclusively for qualitative analysis of virus antigens from infected leaf tissue (Nakane and Pierce, 1966*;* Wicker and Avrameas, 1969). Technical advancements have expanded their use in quantitative analytical procedures (Engvall and Perlmann, 197I, 1972; van Weemen and Schuurs, 1971, 1972).

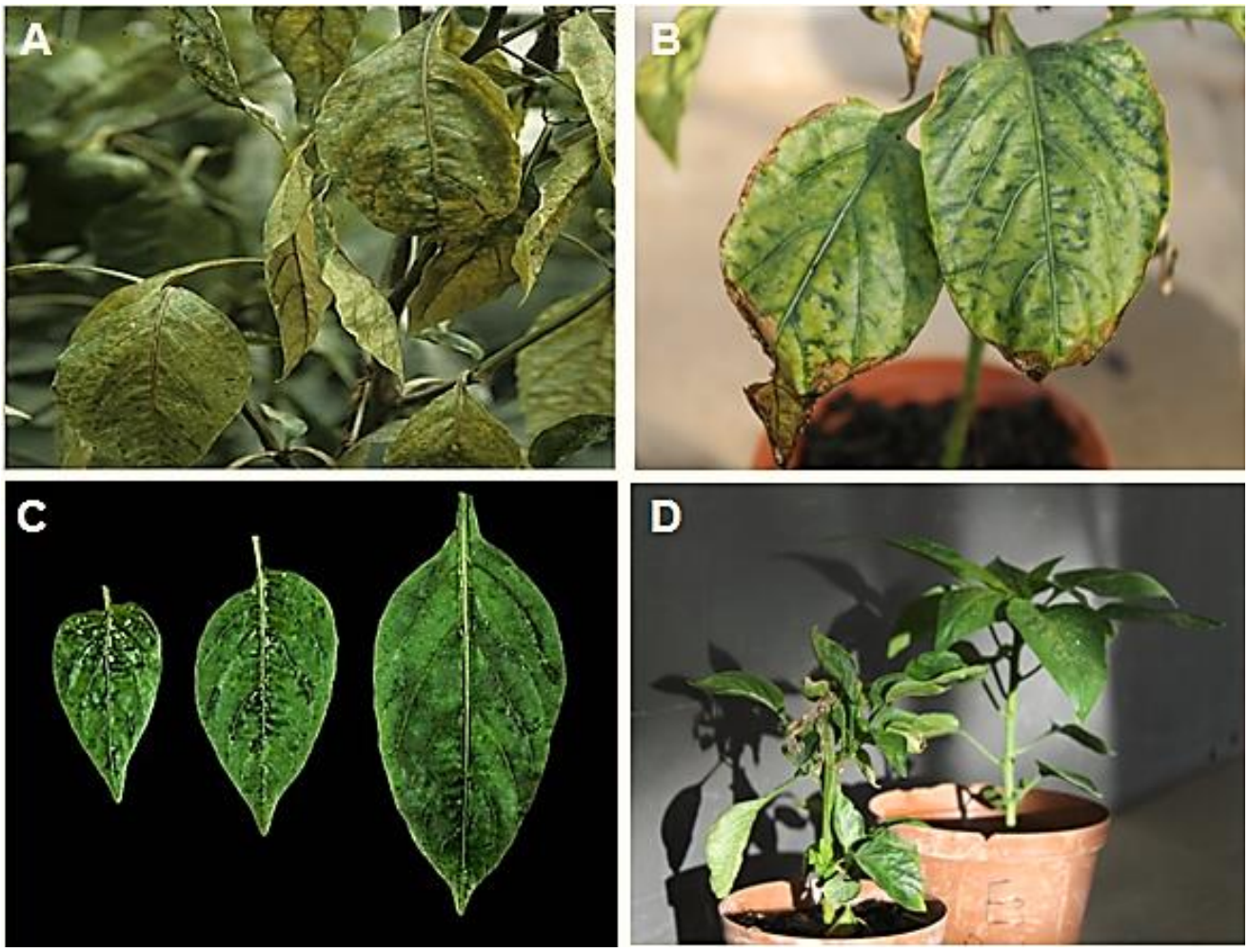

Figure 1.5 PVY symptoms on pepper. A: Vein necrosis and leaf crinkle induced by PVY in pepper (Kerlan, 2006); B: Severe mottle and vein banding in lower leaves (Photo by: Vaneson Moodley, PMB, UKZN. Taken at UKZN Glasshouse Facility. Date: 30/06/2013); C: Mature pepper leaves showing mosaic and dark green vein banding (Cerkauskas, 2004); D: A pepper plant infected with PVY showing stunted growth, death of apical bud and leaf distortion compared to a healthy plant (Photo by: Vaneson Moodley, PMB, UKZN. Taken at UKZN Glasshouse Facility. Date: 30/06/2013).

Due to the strong immunogenic nature of PVY (Shukla *et al*., 1994), the double antibody sandwich (DAS) ELISA (Fig. 1.7) technique is frequently used to detect for the presence of this pathogen (Boonham *et al.*, 2002b). Other widely used approaches include triple antibody sandwich (TAS) ELISA (Fig. 1.6) and antigen coated plate-ELISA (Engvall and Perlmann, 1971). The production of monoclonal antibodies (MAbs) raised against

various isolates of PVY has allowed for the differentiation of the main strain groups i.e. PVY^O, PVY^C and PVY^N on the basis of their coat protein (Gugerli and Fries, 1983). These antibodies have been widely adopted for use in industrial applications. The use of ELISA may produce false positive results. This possibility arises from the fact that recombinant strains of PVY can be serologically identified as PVY^O (due to a PVY^O-like coat protein), but induce PVY^N like symptoms (Nie and Singh, 2002).

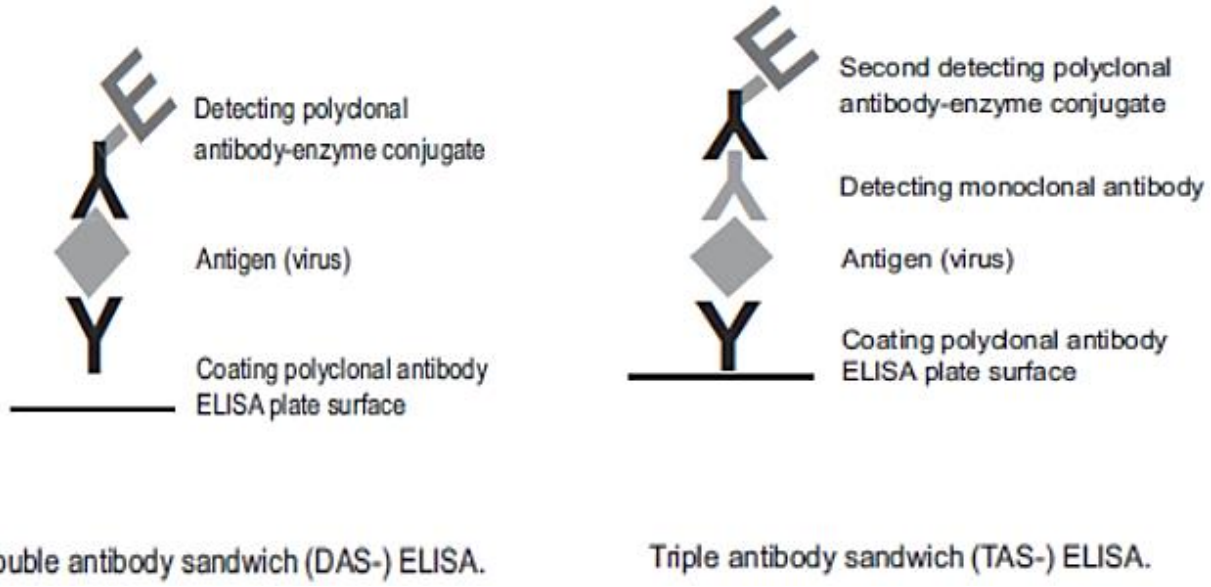

Figure 1.6 Two types of ELISA frequently used to assess for the presence of viruses from diseased plant tissue (Naidu and Hughes, 2003).

1.12.2 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a highly sensitive technique able to successfully detect trace amounts of virus in plant material and insect vectors (Singh and Singh, 1996). Primer design/selection is important to accurately identify the different strains of PVY (Zeng *et al*., 2008). Walsh *et al* (2001) recommends the use of RT-PCR for PVY detection. Genomic data banks allow the selection or design of primers from previously inserted virus nucleotide sequences which target and amplify specific areas on the corresponding genome of interest (Nie and Singh, 2002).

Polymerase chain reaction (PCR) is a three step *in vitro* process (i.e. denaturation, annealing and elongation) which exponentially amplifies the target nucleic acid sequence forming a complex mix of heterologous sequences (Henson and French, 1993; Naidu and Hughes, 2003). Viral RNA present in PVY is reverse transcribed (RT) to produce a complementary DNA strand (cDNA) which is then amplified by PCR (RT-PCR) (Singh and Singh, 1996). Detecting potyviruses using RT-PCR technique has been used with relative success (Langeveld *et al*., 1991; Colinet *et al*., 1994).

1.12.3 Electron microscopy (EM)

The use of electron microscopy (EM) to determine the morphology of infecting particles such as PVY is a common practice in plant pathology research facilities throughout the world (Baker *et al*., 1985; Milne 1993). However, this technique is less useful when trying to detect the presence of virus particles in plant extracts that are ≤ 10^9/ml (Shukla and Gough, 1979). This constraint can be overcome if the virus in the plant sap extract is concentrated prior to visualization.

The detection and differentiation of various rod shaped particles have been successfully accomplished using EM (Boonham *et al*., 2002c). Filamentous/rod shaped virions such as potyviruses and tobamoviruses are more easily distinguishable in negatively stained leaf-dip preparations than icosahedral, geminate and spherical particles (Naidu and Hughes, 2003). The production of unique intracellular inclusion bodies (observed using specific stains) such as cylindrical inclusion bodies produced by viruses belonging to the potyvirus genus can provide sufficient information to identify the disease to a genus level (Edwardson *et al*., 1993).

Immunosorbent electron microscopy (ISEM) is a technique that couples serology and EM which significantly increases the efficacy of virus visualization (Roberts and Harrison 1979). Virus specific antibody-coated grids trap the corresponding virus particles, retaining minimal host debris which is advantageous over the leaf dip method in terms of sensitivity and selectivity (Naidu and Hughes, 2003). This method is also useful to determine the serological relationship between viruses. Although EM and ISEM are both reliable techniques that contribute significantly to pathogen identification and confirmation (particularly when working with smaller numbers of samples), the cost

and maintenance of an EM unit is a limiting factor for many research facilities especially in developing countries (Naidu and Hughes, 2003).

1.12.4 Whole genome sequencing (WGS) and molecular evolution studies

The first reported whole genome sequence (WGS) of PVY by Robaglia *et al* (1986) led to a greater understanding of the genome structure and gene expression of the virus. Sequence comparisons led to an unexpected relationship between potyviruses and other groups of viruses allowing functions to be suggested for the majority of potyvirus genes products (Domier *et al.,* 1987; Lain *et al.,* 1989). The genome structure and gene expression strategy of potyviruses is similar to plant (como- and nepoviruses) and to animal picornaviruses (Riechmann *et al*., 1992). Furthermore, a conserved order of non-structural proteins encoded by a cluster of genes in the genomes of all these viruses has been proposed to function in RNA replication (Riechmann *et al*., 1992). According to Goldbach *et al* (1990), the sequence data allows for the arrangement of these viruses into a supergroup of picornalike plant viruses.

Recombination (the exchange of genetic material among various naturally existing strains/isolates of PVY) is widespread, therefore molecular evolution studies based on the geographical spread and adaptation to new hosts are advantageous for developing better epidemic control strategies (Elena *et al*., 2011; Jones, 2009). The recombination events can be determined from WGS using web based software such as RDP (recombination detection program) (Martin *et al*., 2010). Cuevas *et al* (2012) showed that the diversity of PVY isolates is strongly linked to the host and the geographical origin in a recent study of phylogeography and molecular evolution of PVY whole genomes.

According to Awadalla (2003), most of the references pertaining to recombinant plant-viral genotypes are centred on the analyses of epidemiological sequence data. A major constraint of phylogenetic analysis is the inability to represent an unbiased sample of all the recombination events; instead it typifies successful recombinant genotypes in terms of natural selection or those genotypes that generally induce new pathologies (Elena *et al*., 2011).

1.13 Control of PVY

1.13.1 Conventional methods

The use of yellow sticky polyethylene sheets, course white nets and reflective mulches (repel aphids) are effective physical barriers against the onset of aphid populations (Cohen and Marco, 1973; Cohen, 1981). Moreover, all weeds in the field should be removed prior to planting to reduce aphid populations and sources of inoculum. During the planting season, fields should be monitored for high aphid populations and the incidence of disease. Infected plants should be appropriately sprayed with a pesticide and removed (Cerkauskas, 2005). These physical methods work optimally as part of an integrated pest management system to control the spread of PVY in pepper cultivating regions.

1.13.2 Chemical and vector control

Due to the non-persistent transmission of PVY, the use of insecticides is rendered ineffective unless applied early in the season prior to planting (Cerkauskas, 2005). Several insecticides may heighten aphid activity and the increase the rate of PVY infection (Gabriel *et al*., 1981). Chemical sprays can be applied to weeds surrounding the field which limits the movement of aphids to other plants. Selecting the appropriate planting date is an important criterion to control the spread PVY since early planting may escape high aphid populations that follow later in the season. Moreover, tomatoes and peppers planted early in the season should not be situated near fields where planting will be done later in the season as this creates a source of virus inoculum for subsequent plantings (Cerkauskas, 2005).

Pyrethroids are the most effective type of insecticide against aphids and the spread of PVY, however, the frequent application of pyrethroids may cause a surge in aphid populations (particularly green peach aphids) due to pesticide selection pressure (Nie *et al*., 2013). The foliar application of mineral oil is an effective alternative which delays the onset of PVY infection (Nie *et al*., 2013) by hindering the attachment of virus particles to the aphid stylets during probing (Schramm *et al*., 2011). Bradley *et al*., (1962), reported an approximate 88% reduction of PVY infected crops after foliar application of mineral oil in greenhouse trails. This effect may be reduced in field

applications when new leaves develop between treatments, and if the leaves are not thoroughly covered with the mineral oil during spraying (Boiteau and Wood 1982; Gibson *et al*., 1988).

1.13.3 Sanitary measures

PVY is mechanically transmissible therefore the handling of healthy seedlings should be minimized when disposing of infected plants. Furthermore, farming equipment and apparel (shoes, clothing) used in diseased areas must be thoroughly sterilized prior to use in healthy fields. According to Cerkauskas (2005), this can be accomplished by a) heating or steaming the equipment/apparel for 30 min at 150°C; b) soaking equipment in 1% formaldehyde solution; c) soaking infected apparatus in a 1:10 dilution of a 5.25% sodium hypochlorite solution or d) using a detergent at concentrations stipulated by the manufacturer with all solutions freshly made up to ensure optimal results. Soap, milk or a 3% trisodium phosphate (TSP) solution should be used to wash hands that have been in contact with infected plants (Cerkauskas, 2005).

1.13.4 Resistant varieties

Although numerous control measures have been developed to curb the spread of diseases in agriculture, the use of resistant varieties (where available) still remains the most cost-effective and reliable approach (Kang *et al*., 2005b). Developing crop varieties with the appropriate type of resistance is time consuming and costly however this approach is favored if the resistance is to be durable (Kang *et al*., 2005b). Plants with resistance genes elicit a hyper-sensitive response (HR) upon recognition of a pathogen. HR is an active defense mechanism during which cells surrounding the site of infection die restricting the movement of the virus (visible as a necrotic lesion on leaf/plant surface) (Goldbach *et al*., 2003). The basis of a HR reaction is very likely the result of a gene for gene relationship during which dominant resistant (R) genes in the plant are matched with corresponding avirulence (Avr) genes in the virus. The Nucleotide-binding site plus leucine-rich repeat (NB-LRR) is a type of protein associated with virus resistance and the largest family of resistance (R) genes) (Goldbach *et al*., 2003). The majority virus resistance (R) genes that have been isolated thus far belong to this family (Gururani *et al*., 2012).

The identification of naturally occurring resistance genes is important since they provide a highly efficient barrier to viral infection. Their recessive or dominant inheritance is strongly linked to underlying molecular mechanisms (Robaglia and Caranta, 2006). Although the mechanisms of resistance associated dominant genes are not well understood, they do induce programmed cell death (HR) upon recognition of pathogen-specific components together with an array of signaling pathways which ultimately leads to resistance (Martin, 2003). Resistance to bacteria and fungi is primarily a dominant trait; virus resistance on the other hand is predominantly a result of recessive genes (Robaglia and Caranta, 2006). Most plant infecting viruses encode four to ten proteins and therefore require components from their host during infection. Fraser (1986) suggests that host resistance is linked to mutation or a loss of host components required by the virus for successful infection and replication.

The replication and subsequent movement of potyviruses from the site of infection is achieved via the interaction of viral proteins, host factors and RNA (Kang *et al*., 2005b). Previous studies provide a comprehensive understanding of potyvirus encoded proteins and their role during an infectious cycle, however their interaction with host components required for successful spread remains to be uncovered (Revers *et al*., 1999; Riechmann *et al*., 1992; Urcuqui-Inchima *et al*., 2001). Potyvirus resistance is largely a recessive trait associated with the eukaryotic initiation factor 4E (Ruffel *et al*., 2002).

1.13.5 Breeding

1.13.5.1 Breeding for resistance

Selecting the appropriate strategy in breeding *Capsicum* spp. is dependent on factors associated with the breeding objective as well as the parental lines being used (Greenleaf, 1986). Although resistance to many diseases and pests which attack *Capsicum* spp. has spiked a prodigious effort, intraspecific (different types of pepper within *C. annuum*) as well as interspecific resources (cross-compatible with *C. annuum*) have only recently been exploited by breeders (Pickersgill, 1997). Intraspecific hybridization is extensively used in breeding programs to improve pepper cultivation, particularly with respect to pest and disease resistance (Pickersgill, 1997).

Due to the recalcitrant nature of *C. annuum*, the development of transgenic pepper varieties with virus resistance have been met with limited success. Moreover, chemical, cultural and physical methods to control PVY are not very effective; therefore breeding for resistance is the best alternative to manage PVY disease on pepper. Greenhouse screens revealed sources of PVY resistant pepper varieties in many parts of the world including South America, Europe and South RSA (Pochard *et al*., 1983; Gebre-Selassie *et al*., 1985; Chapter 3 of this dissertation). Breeding techniques for incorporation of PVY resistance genes into commercial pepper genotypes can now be optimized, as information relating to the type of gene action is better understood (Boiteux *et al*., 1996).

Hybridization, backcrossing and pedigree breeding are some of the techniques used in breeding programs to introgress resistance genes into susceptible genotypes of interest (Arnedo-Andres *et al*., 2002). Successive crosses are made between a resistant and susceptible parent line to produce an F_1 hybrid variety. Pathotype specific inoculations or marker-assisted selection (MAS) can be used to identify the desired genotype. There is variation among PVY isolates which makes it difficult to confirm that the inoculated isolate is the appropriate one (Arnedo-Andres *et al*., 2002). Therefore, the availability of molecular markers linked to the *pvr2* locus would be particularly favorable to avoid such drawbacks in breeding programs intended to improve PVY resistant pepper lines (Arnedo-Andres *et al*., 2002).

Staub *et al* (1996) describes numerous methods aimed at developing different types of molecular markers useful for tagging resistance genes. According to Michelmore (2003), understanding the mechanisms by which these resistant genes function together with the signal transduction in host defense pathways is fundamental to the identification of novel resistant alleles and expansion of the gene pool for breeding. Furthermore, resistant genes in breeding progenies can be easily identified on condition that the functional markers are derived from DNA polymorphisms directly responsible for the resistant phenotype (Andersen and Lubberstedt, 2003).

The use of functional markers has spurred a great deal of interest following the increasing number of cloned genes responsible for phenotypic traits and the determination of nucleotide polymorphisms between alleles (Rubio *et al*., 2008).

Interestingly, these functional markers never combine with the trait of interest and remain informative regardless of the recipient genetic background (Anderson and Lubberstedt, 2003). Functional markers are particularly important for MAS of alleles controlling resistance against pathogens and belonging to an allelic series with various resistant specificities (Rubio *et al*., 2008). Thus, the use of a candidate gene approach to clone the *pvr2* followed by molecular and functional characterization of numerous alleles with alternate resistance specificities against potyviruses created the possibility to design functional markers based on allele sequences (Charron *et al*., 2008; Ruffel *et al*., 2002).

MAS is an increasingly popular tool employed in modern plant breeding programs (Yeam *et al*., 2005). The use of indirect selection via molecular genotyping methods facilitates the identification of desired alleles and haplotypes during the initial stages of the plant life cycle and early in the breeding line development which can ultimately lessen or eradicate cycles of phenotypic evaluation (Dubcovsky 2004; Frey *et al*., 2004). MAS is especially significant when the trait is recessively inherited, polygenic or shows low heritability resulting from imprecise phenotype based selection (Yeam *et al*., 2005). Recessive resistance is predominantly expressed in potyviruses which consists of approximately 40% of all known potyviral resistance genes (Provvidenti and Hampton, 1992).

1.13.5.2 Resistance breeding using marker assisted selection

The development of resistant pepper varieties using convention and molecular breeding techniques have been used for a considerable number of years (Kang *et al*., 2005b) during which many resistant genes and quantitative trait loci (QTLs) have been recognized. These include the *pvr1*, *pvr2* and *pvr5* from *C. annuum* situated on chromosome P4 of pepper as well as the *pvr4* from *C. annuum* and *pvr7* from *C. chinense*, both occurring on chromosome P10 of pepper (Kyle and Palloix, 1997; Caranta *et al.*, 1997; Grube *et al.*, 2000; Kerlan, 2006).

A range of genetic markers are currently available to identify resistant genes for use in breeding programs. Several pepper cultivars introgressed with *pvr2*^{1} and *pvr2*^{2} recessive traits have been developed in various parts of the world (Kerlan, 2006).

Additionally, some commercial hybrids have been introgressed with a dominant *pvr4* gene which provides resistance against all PVY strains (Kyle and Palloix, 1997; Kerlan, 2006). Plant breeders continue to search for new genetic sources of resistance that are durable and sexually compatible for exploitation in breeding programs (Grube *et al*., 2000). The pepper industry suffers considerable loss as a result of virus infection and is now becoming the focus of intense genetic and breeding studies previously established for tomato and potato research (Watterson 1993; Pillen *et al*., 1996). The *pot-1* gene from tomato and the *pvr2* gene from pepper that confer complete resistance to PVY have been found to occur in co-linear regions of the tomato and pepper genomes (Parrella *et al*., 2002). Studies conducted by Ruffel *et al* (2005) suggest that these genes use a mechanism that inhibits virus replication in the host tissue. Furthermore, mutations in the central region of the virus genome linked protein (VPg) cistron of PVY is a virulence determinant toward these recessive alleles (Moury *et al*., 2004).

Studies conducted by Ruffel *et al* (2002), indicate that the *pvr2* gene from pepper corresponds with a gene for eukaryotic translation initiation factor 4E (*eIF4E*). Interestingly, the *eIF4E* protein sequences from susceptible cultivars consisting of the *pvr2*$^{+}$ allele differed by two amino acid changes from that of resistant cultivars (*pvr2*^{1} and *pvr2*^{2} alleles) (Ruffel *et al*. 2002). This indicates that potyvirus resistance is controlled by subtle mutations in the *pvr2-eIF4E* locus in pepper. The *pvr2*^{1} and *pvr2*^{2} recessive alleles that confer potyvirus resistance are located at the pvr2 locus in pepper (Kyle and Palloix, 1997). The *pvr2*^{1} allele provides effective resistance against PVY pathotype 0 and the *pvr2*^{2} allele is effective against pathotypes 0 and 1 however, both genes are overcome by the PVY pathotype 1-2 (Ruffel *et al*., 2002).

The *eIF4E* (translation initiation factor) has been recurrently detected in a variety of hosts as a naturally occurring recessively inherited resistance locus (Yeam *et al*., 2005). Genetic mutations occurring within the *eIF4E* initiation factor have been shown to confer resistance to potyviruses in numerous plant species including pepper at the *pvr2* locus (Kang *et al*., 2005b; Ruffel *et al*., 2002). Strong circumstantial evidence suggests that the interaction between *eIF4E* and viral protein VPg is fundamental for determining the outcome of potyvirus–host interactions (Yeam *et al*., 2005). Mutations in the *eIF4E*

occurring at the *pvr2* locus results in gene products that are unable to interact with the viral protein Vpg (Kang *et al*., 2005b). It is therefore possible that functional markers reported in literature are established on biologically meaningful single nucleotide polymorphisms (SNPs) that contribute to the phenotypic characteristic of interest (in this case susceptibility or resistance) (Yeam *et al*., 2005).

There are ten described resistant alleles at the *pvr2* locus, however, four are frequently used in commercial cultivars and are characterized by different resistance specificities against potyviruses such as PVY which affects pepper production regions on a global scale (Rubio *et al*., 2008). The identification, characterization and sequencing of the resistant alleles together with the resistance specificity against various pathotypes of PVY is relative to signature amino acid substitutions arising from a few SNPs within the *elf4E* translation initiation factor (Ruffel *et al*., 2002; Charron *et al*., 2008). Understanding these mechanisms provides the opportunity to design functional markers that will contribute positively toward breeding resistant cultivars specific to the virus and strain that is prevalent in a particular area (Rubio *et al*., 2008).

Rubio *et al* (2008), describes the tetra-primer ARMS-PCR (amplification refractory mutation system – polymerase chain reaction) procedure to identify SNP signatures of four individual alleles at the *pvr2-eIF4E* locus (Fig. 1.7) which regulates pepper resistance to several potyviruses. This method combines a set of four primers in a single PCR reaction to distinguish alleles in homozygous and heterozygous genotypes. The two allele-specific amplifications occur in opposite directions by means of an outer primer and an inner primer. One of the alleles at the SNP is specifically matched by an inner primer which includes a mismatch at position - 2 from the 3' terminus (Ye *et al*., 2001). This procedure allows for the development of allele-specific and codominant markers that can be used to assay large progenies and are particularly beneficial to screen for recessive alleles in heterozygous progenies which cannot be accomplished by means of phenotypic screening (Rubio *et al*., 2008). Furthermore, the primer design allows for the generation of amplicons (different sizes) which can be analyzed by means of agarose gel electrophoresis. The SNPs between alleles can be identified in a single PCR run without further digestion by restriction enzymes, unlike the CAPS (cleaved

amplified polymorphic sequences) markers formerly used for the detection of potyvirus resistant alleles in pepper (Caranta *et al*., 1999; Yeam *et al*., 2005).

The ability to assay plants for multiple alleles at the *pvr2* locus using ARMS-PCR will facilitate breeding for resistance to an array of potyviruses and strains that damage pepper crops globally (Rubio *et al*., 2008). Moreover, this technique eliminates the need for artificial inoculation and enzymatic digestion which consequently reduces the time and cost of genotyping and phenotyping large plant populations (Rubio *et al*., 2008).

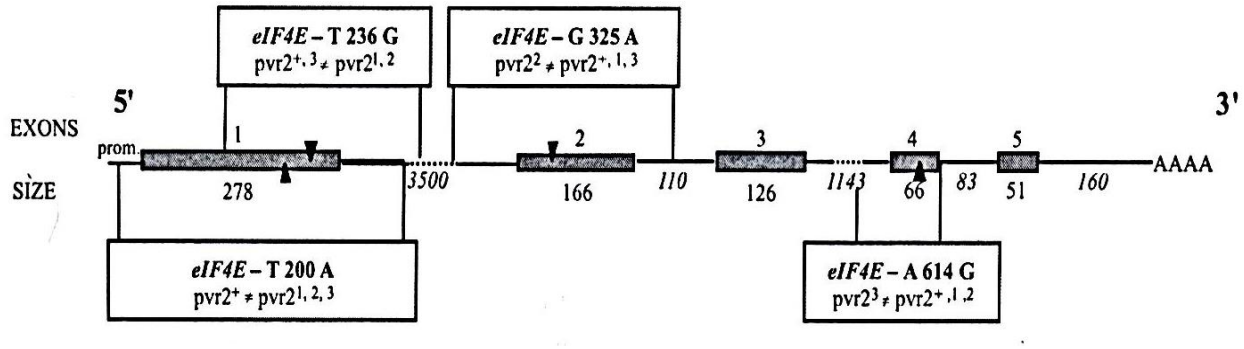

Figure 1.7 SNPs and amplicons of the ARMS-PCR markers located on the pepper *pvr2-elF4E* gene. Exons are represented by large grey bars numbered 1 to 5. Dashed and solid horizontal lines are 5' and 3' UTR and intron regions. Exon and intron sizes are shown beneath the gene frame and the black arrow heads indicate the positions of the SNPs. Large flags depict intervals in which the common and specific amplicons for each primer were generated (Ruffel *et al*., 2004; Rubio *et al*., 2008).

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Chapter 2

Molecular characterization and whole genome sequencing of a South African isolate of Potato Virus Y (PVY) infecting pepper (*Capsicum annuum* L.)

Abstract

Potato Virus Y (PVY) causes serious disease of economic importance in pepper and other major crop species in the family *Solanaceae*. Three major PVY strain groups i.e. O, C, and N have been distinguished on the basis of genome sequencing. In this study, the first full genome sequence of a PVY isolate (JVW-186) infecting pepper from KwaZulu-Natal (KZN), Republic of South Africa (RSA) is reported. The complete genome sequence of JVW-186 was assembled from overlapping RT-PCR clones using MEGA 5 software. Individual ORFs were identified using the nucleotide data base NCBI and aligned using CLUSTALW. RDP4 software was used to identify recombination junctions in the sequence alignment of JVW-186. CLC Main Workbench 6 software was used to determine the nucleotide sequence similarity of recombinant and non-recombinant fragments of JVW-186 in conjunction with ten PVY parental isolates. Phylogenetic trees were constructed from all recombinant and non-recombinant segments of the sequence by the maximum likelihood method using MEGA 5 software. The full length sequence of JVW-186 consists of 9700-bp. Two ORF's were identified at position 186 and 2915 of the sequence alignment encoding the viral polyprotein and the frameshift translated protein P3N-PIPO respectively. RDP4 software confirmed three recombination breakpoints at position 343, 1365 and 9308 of the sequence resulting in four segments of the genome. At each recombination event, a 1021-bp fragment at the 5' end in the region of the P1/HC-Pro protein and a 392-bp

fragment in the region of the coat protein shared a high sequence similarity of 91.8 % and 98.89 % to the potato borne PVY^{C} parental isolate PRI-509 and the PVY^{O} parental isolate SASA-110 respectively. Phylogenetic analysis of recombinant fragments 2 and 4 clustered within the C and O clades respectively. The non-recombinant fragment 1 clustered within the C clade of PVY isolates; however the large 7942-bp fragment 3 did not cluster within any of the clades. This suggests the possibility of a novel strain group that has evolved due to the dynamics of selection pressure or the likelihood of an ancestral PVY strain. Since resistance to PVY in pepper is mediated by a series of pathotype specific recessive alleles at the *pvr2-eIF4E* locus, identification of the JVW-186 pathotype will aid in the development of resistant pepper varieties.

2.1 Introduction

Potato virus Y (PVY), the type member of the Potyvirus genus in the family Potyviridae is an important pathogen of pepper (*Capsicum annuum* L.) and other solanaceous crops (Shukla *et al*., 1994). The monopartite PVY genome comprises a single-stranded, positive-sense RNA molecule about 9.7-kb in length (Shukla *et al.*, 1994). PVY virions are non-enveloped, filamentous, flexuous rods that are approximately 740-nm long and 11-nm wide (Shukla *et al.,* 1994). The definitive morphological structure consists of approximately 2000 copies of coat protein (CP) that forms a cylindrical inclusion body (CIb) which is reflected as a key phenotypic criterion for distinguishing a potyvirus infection from other virus groups (Talbot, 2004). The viral RNA contains a single extended open reading frame which is encapsidated by the cylindrical inclusion body (CIb) and has a non-translated 5' terminal region (5'-NTR) together with a 3' poly-A tail which acts directly as mRNA (Dougherty and Carrington, 1988; Van der Vlugt *et al.,* 1989). A small open reading frame (ORF) known as PIPO (pretty impressive potyvirus ORF) which is generated by a +2 frameshift was recently discovered by Chung *et al* (2008). PIPO overlaps with the P3 coding region of all members of the potyvirus family and encodes an approximate 7 kDa protein. P3N-PIPO is a previously unrecognized potyvirus protein implicated in cell to cell movement of the virus (Vijayapalani *et al*., 2012).

The 5'-NTR mediates cap independent translation and comprises approximately 144 nucleotides that has a considerably higher adenine to guanine ratio, and is associated with a viral genome linked protein (VPg) (Carrington and Freed, 1990). The VPg which functions to enhance transcription is also a unique virulence determinant toward overcoming resistance genes in pepper (Moury *et al*., 2004). The viral RNA encodes a large single (350 kDa) polypeptide which is cleaved by three viral proteases (NIa, HC-Pro and P1) which undergoes co-translational and post-translational cleavage yielding ten functional proteins (Basso *et al.,* 1994). These proteins include, P1 (P1 Protein; 34-64 kDa), HC-Pro (Helper Component Proteinase; 50 kDa), P3 (P3 Protein; 37 kDa), 6K1 (6 kDa Protein 1), CIb (Cylindrical inclusion body; 70 kDa), 6K2 (6 kDa Protein 2), VPg (Viral Genome-linked Protein; 25 kDa), NIa-Pro (Nuclear Inclusion Protein a, Proteinase domain; 49 kDa), NIb (Nuclear Inclusion Protein b; 58 kDa) and the CP (Coat Protein; 30 kDa) (Talbot, 2004).

PVY strains comprise a wide variety of genetically diverse isolates (Singh *et al*., 2008). These naturally occurring strain groups have been classified as PVY^{O} (common or ordinary strain), PVY^{N} (tobacco veinal necrosis strain) and PVY^{C} (stipple streak strain) according to serology, molecular assays and symptomology (Karasev *et al*., 2010). The first described PVY isolate, in the early 1930s, was assigned to the non-necrotic PVY^{O} group which has remained predominant among PVY isolates gathered during field surveys (Smith, 1931). Recent studies indicate that the balance between necrotic and non-necrotic isolates has since changed, with a higher incidence of necrotic isolates now present in natural populations (Piche *et al.*, 2004; Lindner and Billenkamp, 2005; Rolland *et al*., 2009).

Extensive studies to determine the genetic diversity of potyviruses have been predominantly based on the coat protein gene (Shukla *et al*., 1994). An estimated 8% variation has been established along the entire 9.7 kb PVY genome following alignment of complete genome sequences of numerous PVY strains belonging to the PVY^{O} and PVY^{N} groups (Karasev *et al*., 2011). More-over, a multitude of recombinants possessing spliced fragments of PVY^{O} and PVY^{N} sequences in their genomes have been identified in addition to these main parental genomes (Karasev *et al*., 2011).

Reports of numerous recombinant and non-recombinant isolates of PVY have been described from many parts of the world including RSA (Kerlan, 2006, Ibaba and Gubba, 2011). The recombinant strains include the PVY^{N}-W, PVY^{NTN} and $PVY^{N:O}$. According to Thompson (1997), PVY^{O} and PVY^{N} are the most commonly identified strains in RSA. The level of damage to crops infected with PVY is collectively determined by the stage of plant growth during which infection occurs, the type of infecting strain, the degree of resistance/susceptibility of the plant toward the infecting pathotype and the viral load (Warren *et al*., 2005).

Pathotypes of PVY that infect pepper have been classified as 0, 1 and 1-2 based on their ability to overcome the recessive alleles (*pvr2*$^{+}$, *prv2*^{1} and *pvr2*^{2}) at the *prv2* resistance locus (Gebre-Selassie *et al.*, 1985). Furthermore, 'common' and 'necrotic isolates within these pathotypes have been defined (d'Aquino *et al.*, 1995). Serological studies indicate that isolates of PVY infecting pepper are closely related to the PVY^{O} strain group; however no relationship has been established between serotypes and pathotypes (Soto *et al*., 1994). While molecular studies of the coat protein amino acid sequences have proven useful in the taxonomy of potyviruses, it was unable to differentiate pathotypes of PVY infecting pepper (Llave *et al*., 1999). The PVY^{C} lineage can be divided into two sub-groups denoted C1 and C2. Isolates belonging to the C1 group are termed non-potato isolates which are able to infect both pepper and potato; the C2 group consists of potato infecting isolates that infect pepper with reduced efficiency (via aphid transmission) but not mechanically (Blanco-Urgoiti *et al.*, 1998; Romero *et al.*, 2001). The incidence of PVY^{C} has been reported in Europe, Australia/New Zealand, the Americas, and RSA (De Bokx and Huttinga, 1981; Brunt, 2001). Although strains infecting pepper display a strong level of host specificity (Gebre-Selassie *et al.*, 1985; McDonald and Kristjanson, 1993; d'Aquino *et al.*, 1995), numerous isolates from potato and tobacco have been reported to infect pepper and tomato (McDonald and Kristjanson, 1993; Stobbs *et al*., 1994; Legnani *et al.,*1995). According to Kerlan (2006), the vast majority of PVY isolates are capable of infecting tobacco and tomato; however potato and pepper are more pathotype specific.

The use of genetic resources to manage PVY disease on pepper is dependent upon identification of the infecting strain/pathotype in a particular area. Knowledge of the mechanisms involved during resistance breakdown is essential to developing pepper varieties with durable resistance. According to Ayme *et al* (2006), genetic variation of PVY isolates as a result of evolution (avirulent to virulent) is the first step in resistance breakdown. Therefore, molecular evolution studies relative to the extremely pervasive nature of recombination among naturally occurring PVY isolates is necessary (Cuevas *et al*., 2012). The probability for rapid evolution among genetically diverse populations of RNA viruses such as PVY is the likely cause of their wide distribution and the ability to adapt to changing environmental conditions throughout the world (Domingo *et al*., 1996). The large-scale cultivation of hybrid varieties introgressed with genetic resistance in plant breeding programs may be easily overcome by the adaptation of these virus populations (Pelham *et al*., 1970).

Pathogenicity among PVY isolates is associated with the variety of strains in natural populations; however, recombinant forms are responsible for the major losses incurred by the global agricultural industry (Visser *et al*., 2012). Against this background, the aim of this study was to molecularly characterize and sequence the whole genome of a PVY isolate infecting pepper in KZN. Information generated from this study will contribute towards effectively managing PVY related diseases of pepper in KZN.

2.2 Materials and Method

2.2.1 Virus isolate

The isolate of PVY used in this study was obtained from a previous field survey of symptomatic pepper crops across the KZN province, RSA (Ibaba and Gubba, 2011). The dehydrated PVY isolate was propagated on the laboratory host *Nicotiana tabacum* cv. Xanthi in an insect-proof greenhouse and symptomatic leaves were harvested and stored at -80°C until further use.

2.2.2 Virus purification

PVY was purified from *Nicotiana tabacum* cv. Xanthi leaves according to the method described by Van Oosten (1972) with some amendments: 100 g of infected leaf material was finely crushed using a mortar and pestle in liquid nitrogen and homogenized in 100 mM sodium citrate buffer (pH 8.3) containing 20 mM sodium sulfite and 2 mM EDTA (1:3 w/v). The homogenate was filtered through cheesecloth and the subsequent filtrate was centrifuged at 10 000 x *g* for 10 min. Three percent (v/v) Triton X-100 was added to the total volume of the supernatant and mixed on ice for 1 h. After centrifugation (Beckman Coulter, Avanti, J-26 XP1) at 75 000 x *g* for 2 h, the pellet was re-suspended in 10 mM sodium citrate buffer (pH 8.3) containing 500 mM urea and 0.1% (v/v) 2-mercaptoethanol in a shaking incubator at 4°C overnight. The suspension was layered onto a 30% (w/v) sucrose cushion (ice cold) in 0.01 M sodium citrate buffer (pH 8.3) and centrifuged at 75 000 x *g* for 2 h. The pellet was re-suspended in 10 mM sodium citrate buffer and transferred to a 2 ml micro-centrifuge tube. The sample was clarified by centrifugation at 13 000 x *g* for 5 min at room temperature and viewed using a JOEL JEM-1400 transmission electron microscope after staining with uranyle acetate.

2.2.3 Analysis of PVY coat protein using SDS-PAGE

The size of the coat protein of the purified PVY sample was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The coat protein from the purified virus preparation was dissociated and resolved by electrophoresis on a 12.5% SDS-PAGE gel, using a Hoefer mini VE vertical electrophoresis system. A detailed description of each solution used in the preparation of the running and stacking gels are presented in Appendix A. The volume of each solution (Appendix A) required for preparation of the stacking and resolving gels are listed in Table 2.1. The purified virus sample was added to reducing treatment buffer (Solution G) at a ratio of 1:1 and denatured at 100°C for 3-min on a heating block before loading it onto the gel. A PageRuler prestained protein ladder (10-170 kDa; Thermo Scientific, USA) was used to size and monitor protein migration during SDS-PAGE. Proteins were visualized by submerging the gel in Aqua Stain (Aquascience, UK) for 30 min on an orbital shaker at low speed and photographed using a Syngene snapshot gel documentation system.

Table 2.1 Solutions used for preparation of running and stacking gels (Laemmli, 1970).

Solution	12.5% Resolving gel (ml)	4% Stacking gel (ml)
A (Monomer solution)	6.25	0.94
B (4x Running gel buffer)	3.75	0
C (4x Stacking gel buffer)	0	1.75
D (10% (w/v) SDS)	0.15	0.07
E (10% (w/v) APS)	0.075	0.035
Distilled water	4.75	4.3
TEMED	0.0075	0.015

2.2.4 RT-PCR assays

The complete genome sequence of PVY was amplified using a range of primers listed in Table 2.2. Primers were designed to cover the gaps in the alignment from previously amplified regions of the genome using the VPg-F/VPg-R, P3-F/P3-R and the S-Primer/M4T primer pairs. MEGA 5 (Tamura *et al*., 2011), Primer 3 (Rozen and Skaletsky, 1998) and NetPrimer (www.premierbiosoft.com) software were collectively used for optimal primer design. The purified PVY sample was used as a template in the reverse transcription (RT) procedure. RT was performed using a Revert Aid Reverse Transcriptase kit (Thermo Scientific, USA) according to the manufacturers' instructions. cDNA was obtained from 10 µl of purified virus (initially heated at 65°C for 5 min) using gene specific primers in a 20 µl reaction. The reverse transcriptase step was performed at 42°C for 1 h and terminated at 70°C for 10 min. PCR was performed using KAPA2G Fast DNA Polymerase (Kapa Biosystems, South Africa) according to the manufacturers' instructions in a 25 µl reaction with 3 µl of cDNA. The annealing temperature for the primer sequences used in this study together with their expected band sizes have been summarized in Table 2. PCR cycling conditions for amplification were 95°C for 2 min; 35 cycles of 20 s at 95°C, 30 s at the primer specific annealing temperature (Table 2.2) and 35 s at 72°C; and a final elongation step of 72°C for 5 min. Bands were resolved on a 1.5% agarose gel stained with SYBR safe.

Table 2.2 Primers used to amplify and clone regions of the PVY isolate JVW-186.

Primer	Sequence 5'-3'	Tm (°C)	Amplicon (bp)	Reference
VPg-F	GAATYCAAGCHYTRAAGTTTCG	58	547	Ben Khalifa *et al*., 2009
VPg-R	GCTTCATGYTCYACHTCCTG			Ben Khalifa *et al*., 2009
P3-F	TCACCNTTYAGAGARGGNGG	60	1145	Ben Khalifa *et al*., 2009
P3-R	CARTCRCTCCTTTCAGCATC			Ben Khalifa *et al*., 2009
S-Primer	GGNAAYAAYAGYGGNCARCC	57	1700	Chen *et al*., 2001
M4T	$GTTTTCCCAGTCACGAC(T)_{15}$			Chen *et al*., 2001
F1476	GCTCRTGGATGGCAGRTGGC	62	1100	This study
P3-2490	GYGCTGCYGACTCAGACATTAT			This study
F7093	AGCATGTDGTTAAAGGAGARTGTCG	62	1000	This study
NIb-8045	CGTGCTGTCWWTCTCTTCAAAATCG			This study
F7093	AGCATGTDGTTAAAGGAGARTGTCG	62	1000	This study
NIb-8073	AGTCATCGCCATTKACAAAGAACAC			This study
JAC PVY5'	GGTGGAAATTAAAACAACTCCCTAC	60	1300	This study
PVY3-1200	AYTGTTGRGCACAGGTRGGGC			Schubert *et al*., 2007

Primer	Sequence 5'-3'	Tm (°C)	Amplicon (bp)	Reference
P3-3470	ACAAATGGAGGAATACGATGTGCGAC	64	850	This study
Y3-4270	CRACYTCYCTTCCCACTGGAG			Schubert *et al*., 2007
P3-3533	AGTGGTAGCATTTATGGCTTTGGTGA	63	800	This study
Y3-4270	CRACYTCYCTTCCCACTGGAG			Schubert *et al*., 2007
Y5-3000	CAACATGGYAYTCATACARAGCAAA	62	1300	Schubert *et al*., 2007
Y3-4270	CRACYTCYCTTCCCACTGGAG			Schubert *et al*., 2007
P3-3533	AGTGGTAGCATTTATGGCTTTGGTGA	63	2300	This study
VPg-5759	ARCCAGCCCTYTTGTCRCGAGCA			This study
VPg-6202	ACTTTGAAYTRAGGCARACTGGRCC	63	1300	This study
Y3-7560	TTYTTGCCWCCATACATRGCTCC			Schubert *et al*., 2007
VPg-6202	ACTTTGAAYTRAGGCARACTGGRCC	62	1900	This study
NIb-8045	CGTGCTGTCWWTCTCTTCAAAATCG			
Y5-1049	GAATTTTTGGGATGGTCTGGACGG	62	1100	This study
Y3-2492	AAGTGCTGCCGACTCAGACATTAT			This study

2.2.5 Cloning and sequencing

PCR amplicons of the expected size were excised from the agarose gel and purified using the MinElute Gel Extraction kit (Qiagen, Netherlands). The purified product was cloned into the pCR®2.1 vector and transformed into competent *E.coli* (TOP10) cells using a TA Cloning kit (Invitrogen, CA) following the manufacturers' guidelines. Plasmid DNA was extracted from single white colonies selected on Luria Bertani (LB) plates containing 50µg/ml kanamycin and 40mg/ml X-Gal using a QIAprep Spin Miniprep Kit (Qiagen, Netherlands). Inserts were validated by restriction analysis using the endonuclease EcoRI (Thermo Scientific, USA). Four clones from each PCR product were sequenced at Inqaba Biotec (Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa) in the forward and reverse directions using the M13 primers. The genome was constructed from overlapping RT-PCR clones.

2.2.6 Sequence and recombinant analysis

The nucleotide sequences of the South African isolate of PVY infecting pepper (JVW-186; GenBank accession number KF770835) was analysed in comparison with sequence data from 10 known parental isolates of PVY selected from GenBank. Details of each PVY^{O}, PVY^{N} and PVY^{C} parental isolate used for the sequence anaylsis were obtained from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nml.nih.gov) (Table 2.3). Sequences were aligned using MEGA 5 (Tamura *et al*., 2011) and CLUSTALW (Thompson *et al*., 1994) software with a final dataset composed of 9700 nucleotides for the complete genome elucidation. Segmental recombination events of the complete genome sequence was identified using Recombination Detection Program 4 (RDP4) software (Martin *et al*., 2010). Only recombination breakpoints detected by a minimum of three methods within the program were considered as significant data. CLC Main Workbench 6 (www.clcbio.com) software was used to determine the nucleotide sequence similarity of recombinant and non-recombinant fragments of JVW-186 in conjunction with ten PVY parental isolates. Phylogenetic analysis was independently conducted on the resulting recombinant and non-recombinant sequence data to evaluate the degree of recombination.

Table 2.3 Parental PVY isolates obtained from the NCBI nucleotide database used for phylogenetic analysis in conjunction with JVW-186.

Isolate	Strain	Accession No.	Reference
PRI-509	C	EU563512	Dullemans *et al*., 2011
SON41	C	AJ439544	Moury *et al*., 2002
LYE84.2	C	AJ439545	Moury *et al*., 2002
Adgen	C	AJ890348	Schubert *et al*., 2007
SCRI-O	O	AJ585196	Barker, 2003
SASA-110	O	AJ585195	Barker, 2003
Mont	N	AY884938	Lorenzen *et al*., 2006
CH-605	N	X97895	Jakab *et al*., 1997
SASA-61	N	AJ585198	Barker and McGeachy, unpublished data
RRA-1	N	AY884984	Lorenzen *et al*., 2006

2.2.7 Phylogenetic analysis

Phylogeny was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree was rooted using Sunflower chlorotic mottle virus (SuCMoV) (GU181199) as an outgroup sequence for the phylogenetic analysis because it is closely related to PVY and is considered to be a distant relative of PVY (Moury, 2009). Phylogenetic trees were constructed from the multiple sequence alignment data generated from CLUSTALW using the software MEGA 5. The bootstrap values are indicated next to the branches in which the associated taxa cluster. The percentage value at each node was assessed using 1000 bootstrap replicates.

2.3 Results

2.3.1 Transmission electron microscopy (TEM)

Figure 2.1A shows a high concentration of aggregated PVY virions from which the actual size of individual virus particles are not easily determined. Aggregation of virus particles is typically associated with potyviruses. Protein induced aggregation results from the interaction of the virus with the abundance of polyphenolic compounds, proteins and pigments found in the host tissue (Rupar *et al*., 2013). Figure 2.1B shows a suspension of flexuous filamentous rods approximately 700 nm in length and 11 nm wide after a ten-fold dilution of the original sample (Fig. 2.1A)

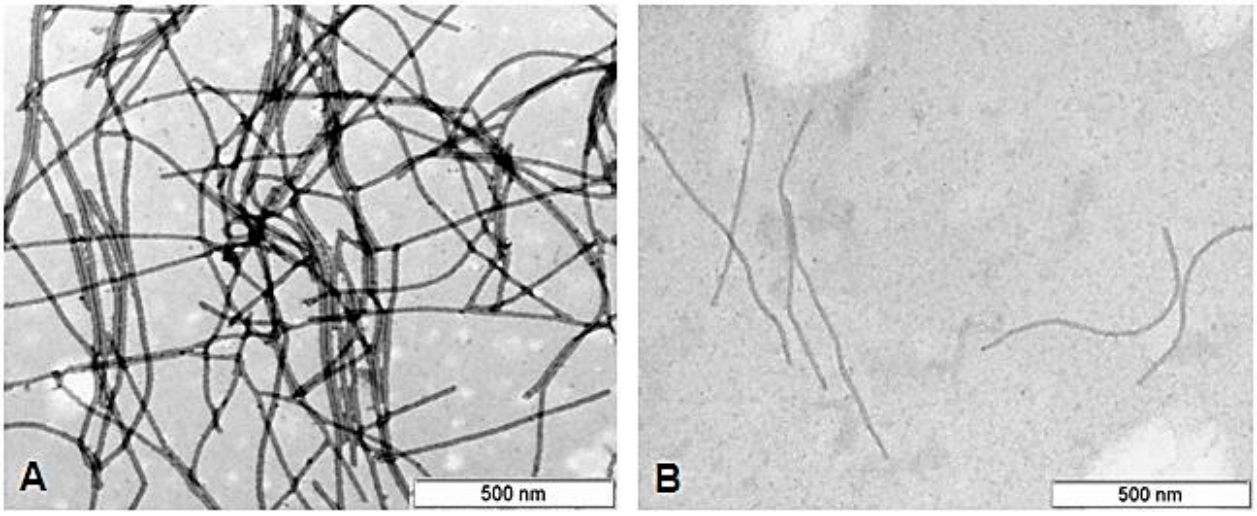

Figure 2.1 Electron micrograph of a negatively stained purified suspension of PVY virions (magnification: 40 000x). (A) Protein induced agglutination of PVY. (B) Ten-fold dilution of purified PVY preparation.

2.3.2 Evaluation of virus purity by SDS-PAGE

The SDS-PAGE profile of the purified virus shows a single 30 kDa band (Fig. 2.2) which corresponds to the PVY multifunctional coat protein gene involved in virus assembly, aphid transmission and local and systemic movement (Urcuqui-Inchima *et al.*, 2001). Furthermore, the single band in lane 1 provides evidence that the purified PVY sample was devoid of other virus particles with different coat protein sizes.

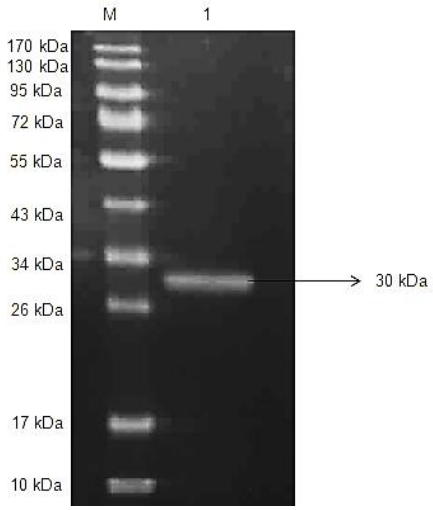

Figure 2.2 SDS-PAGE analysis of a purified sample of PVY. Lane M: Protein Ladder (Thermo Scientific); Lane 1: Purified PVY sample showing a single 30 kDa band.

2.3.3 Identification of ORFs

The complete genome sequence of JVW-186 consisted of 9700-bp [3021 adenine (A), 1776 cytosine (C), 2264 guanine (G) and 2639 thiamine (T)] (Appendix B). Individual ORFs were identified using the nucleotide data base NCBI and aligned with CLUSTALW. Position 1 to 185 of the nucleotide sequence aligned with the 5' NTR (non-translated region) of PVY. The ORF at position 186 encodes a single large polyprotein (186 - 9371) which is cleaved by three viral proteases to form ten functional proteins (Basso *et al*., 1994) (Table 2.4). A second ORF (PIPO) at position 2915 generated by a +2 reading frame overlaps with the P3 coding region (2915 - 3145) of JVW-186 encoding the P3N-PIPO. Position 9372 to 9700 aligned with the 3' NTR of PVY. A percentage comparison of individual protein products (Table 4) revealed high levels of sequence similarity (> 90%) in the CI, VPg, NIa, Nib and the CP coding regions of all PVY isolates used in this study. In addition, the 6K1 protein of JVW-186 shared 100% similarity with PRI-509, SON41 and SASA110. A full comparison of each protein product together with the nucleotide sequence similarity of the non-coding 5’ NTR and 3’NTR of the isolates used in this study are summarized in Table 2.5.

Protein analysis revealed conserved motifs at various regions of the JVW-186 sequence alignment. A single amino acid substitution from Lys (K) to Met (M) at aa 50 in the HC-

Pro N-terminal region of JVW-186 was observed at the highly conserved tetrapeptide Lys-Ile-Thr-Cys (KITC) motif (Sasaya *et al*., 2000) which corresponds to aa 50 to 53. Additionally, SON41 was the only PVY parental isolate with the same amino acid change in this region of the genome. The Pro-Thr-Lys (PTK) motif in the C-terminal region (aa 308 – 310) and the Phe-Arg-Asn-Lys (FRNK) motif (179-182) in the central domain of the HC-Pro (Ala-Poikela *et al*., 2011) were conserved in all PVY isolates used in this study. The amino acid change from Ala (A) to Ser (S) was observed at aa 247 in the central part of the conserved motif Leu-Ala-Ile-Gly-Asn (LAIGN) (aa 246 – 250) (Ala-Poikela *et al*., 2011). The GDSG motif (Barret, 1986) at aa 233 – 246 of the P1 protease, the DAG motif (aa 6 – 8) near the N-terminal of the coat protein (Atreya *et al*., 1990), the GDD motif within the NIb (aa 352 – 354) typically associated with RNA-dependent RNA polymerase (RdRp) super group I (Hong and Hunt, 1996) and the RKK motif (aa 41 – 43) an NTP-binding site (Grzela *et al*., 2006) in the VPg were highly conserved in all the PVY parental isolates including JVW-186.

Amino acid alignment of the 23 codon long VPg central domain of JVW-186 in conjunction with a range of PVY isolates which corresponds to aa 101 – 123 revealed five unique amino acid residues i.e. L (aa 113), V (aa 115), G (aa 119), N (aa 120) and T (123) (Fig. 3). The PVY isolates used for comparison of the VPg region were selected according to a recent study conducted by Ben Khalifa *et al* (2012) in which these isolates were grouped according to their pathogenicity. Amino acid residues in the VPg central region that correspond with PVY isolates GHA3 and SE15 (Fig. 2.3) belong to pathotype (0,1,3) and all other isolates including JVW-186 belong to pathotype 0 (Ben Khalifa *et al*., 2012).

Table 2.4 Identification of 10 coding regions in the sequence alignment of JVW-186 using NCBI.

Coding Region (bp)	Product (Protein)
186 - 1040	P1
1041 - 2504	Helper Component-Protease (HC-Pro)
2406 - 3500	P3
3501 - 3656	6K1
3657 - 5558	Cytoplasmic Inclusion (CI)
5559 - 5714	6K2
5715 - 6278	Virus Genome-Linked Protein (VPg)
6279 - 7010	Nuclear Inclusion a (NIa)
7011 - 8573	Nuclear Inclusion b (NIb)
8574 - 9368	Coat Protein (CP)

2.3.4 Recombinant analysis

RDP4 analysis revealed two recombination events consisting of spliced fragments from two PVY parental isolates SON41 (AJ439544) and SASA-110 (AJ585195). The breakpoints were estimated at position 343, 1365 and 9308-bp in the alignment resulting in four segments of the genome. Each recombinant and non-recombinant segment of the sequence alignment for the isolate JVW-186 (Fig. 2.4) was independently characterized using phylogenetic analysis. The fragments are characterized as follows: Fragment 1: non-recombinant segment at position 1-342 (342-bp); Fragment 2: recombinant segment at position 343-1364 (1021-bp); Fragment 3: large non-recombinant segment at position 1365-9307 (7942-bp) and Fragment 4: recombinant segment at 9308-9700 (392-bp). The first recombinant breakpoint was detected by four methods implemented in RDP4 i.e. Boot Scan (1.765×10^{-04}), Max Chi (5.586×10^{-06}), Chimaera (1.420×10^{-07}) and SiScan (8.261×10^{-07}) which confirmed the recombination event. The second recombination event was confirmed by 5 methods i.e. GENECONV (4.960×10^{-07}) Boot Scan (7.627×10^{-10}), Max Chi (1.288×10^{-03}), Chimaera (2.458×10^{-02}) and SiScan (1.808×10^{-06}) implemented in RDP4.

Table 2.5. Sequence similarity of non-coding and coding regions of JVW-186 in conjunction with 10 PVY parental isolates using CLC Main Workbench 6 software.

	Non-coding Regions (%)		Coding Regions (%)										
Isolate	**5'NTR**	**3'NTR**	**P1**	**HC-Pro**	**P3**	**6K1**	**CI**	**6K2**	**VPg**	**NIa**	**NIb**	**CP**	**PIPO**
JVW-186	100	100	100	100	100	100	100	100	100	100	100	100	100
PRI-509	86.49	94.22	89.44	94.30	92.05	100	96.53	90.38	95.74	95.08	96.16	94.78	87.18
Adgen	85.41	91.49	83.45	93.86	92.60	96.15	95.90	92.31	94.68	93.44	95.79	94.4	84.62
LYE84.2	88.11	93.92	85.21	93.64	90.68	98.08	95.90	86.54	94.15	93.44	95.78	95.15	85.90
SON41	84.32	93.01	87.32	94.52	91.78	100	96.21	88.46	93.09	93.85	95.97	95.15	84.62
SASA-110	64.32	92.71	81.69	93.20	91.78	100	95.90	88.46	93.09	90.98	95.78	93.28	80.77
SCRI-O	78.92	97.87	80.99	92.76	91.23	94.23	95.74	88.46	92.02	90.16	94.63	94.03	79.49
SASA-61	52.38	79.64	73.59	89.91	89.32	86.54	95.43	88.46	92.55	90.16	93.67	94.78	71.79
RRA-1	68.25	82.67	73.24	90.13	89.59	86.54	95.43	90.38	92.55	91.39	93.86	94.03	71.79
CH-605	67.20	85.71	71.48	90.35	89.32	84.62	95.43	90.38	93.62	90.98	93.86	94.03	73.08
Mont	66.14	86.02	71.83	90.57	89.32	86.54	95.58	86.54	93.09	90.57	94.24	94.78	73.08

- Non-coding regions – nucleotide sequence similarity
- Coding regions – protein sequence similarity

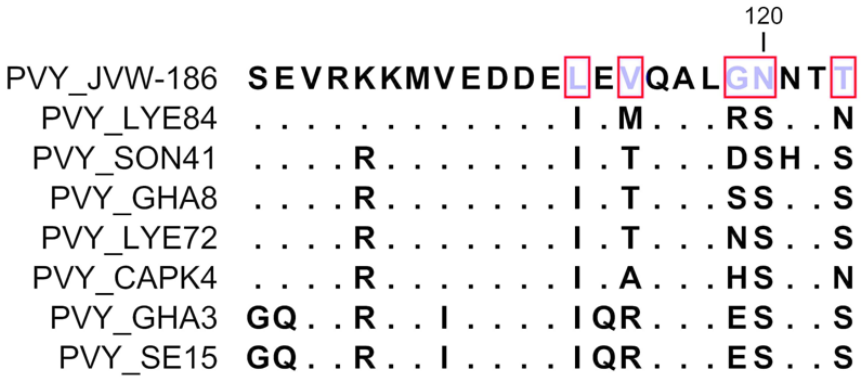

Figure 2.3 Analysis of amino acid residues in the VPg central domain (aa 101 – 123).

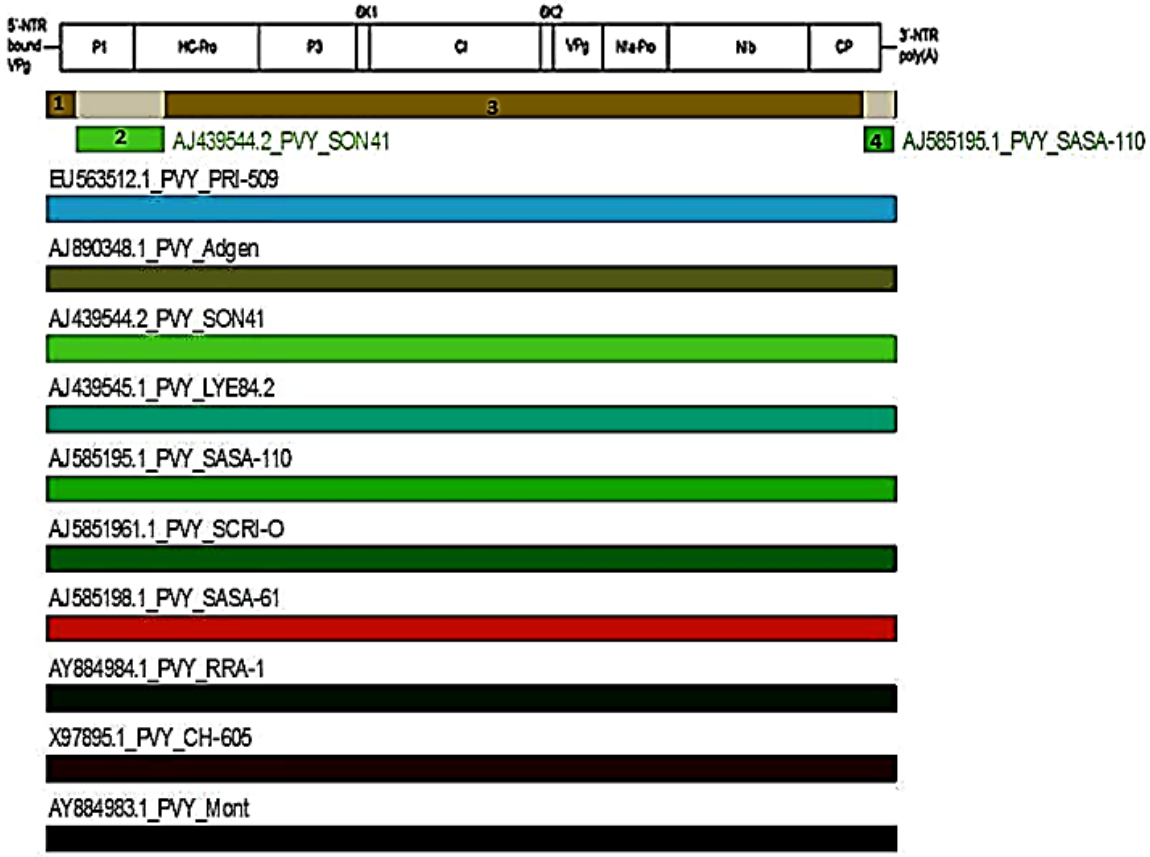

Figure 2.4 Recombination events occurring at two breakpoints of the sequence alignment for the pepper infecting PVY isolate (JVW-186) identified using RDP4 analysis. Ten parental isolates of PVY were used to generate the data.

2.3.5 Nucleotide sequence analysis

The non-recombinant 342-bp fragment positioned at the 5' NTR/P1 coding region of the JVW-186 sequence alignment shared a high sequence similarity (83.87%) to the PVY^{C} parental isolate LYE84.2. along with PRI-509 (82.70 %) and Adgen (81.23 %) (Fig. 2.5).

The recombinant breakpoint occurred at position 343 in the P1 coding region of the JVW-186 sequence alignment. The 1021-bp recombination event from position 343-1364 overlaps into the HC-Pro coding region. Although RDP4 analysis indicated that the recombination event closely resembled a part of the genome similar to the PVY^{C} parental isolate SON41, nucleotide sequence analysis revealed a higher sequence similarity (98.1%) to PVY^{C} parental isolate PRI-509 (Fig. 2.6).

		1	2	3	4	5	6	7	8	9	10	11	12
JVW-186	1		82.70	81.23	83.87	79.77	68.91	76.54	58.55	66.09	65.51	65.22	43.88
PRI-509	2	82.70		88.56	92.38	91.50	73.53	80.88	59.59	68.31	66.96	67.15	43.88
Adgen	3	81.23	88.56		87.39	84.75	75.95	84.46	61.74	71.01	70.14	70.43	43.62
LYE84.2	4	83.87	92.38	87.39		87.98	72.43	81.23	60.58	68.70	68.70	68.41	43.11
SON41	5	79.77	91.50	84.75	87.98		73.61	80.94	60.58	68.12	66.96	65.80	43.11
SASA-110	6	68.91	73.53	75.95	72.43	73.61		88.24	67.09	62.21	60.58	61.05	37.24
SCRI-O	7	76.54	80.88	84.46	81.23	80.94	88.24		62.50	70.93	69.28	69.48	43.11
SASA-61	8	58.55	59.59	61.74	60.58	60.58	67.09	62.50		89.83	78.84	78.20	38.78
RRA-1	9	66.09	68.31	71.01	68.70	68.12	62.21	70.93	89.83		86.96	86.92	44.64
CH-605	10	65.51	66.96	70.14	68.70	66.96	60.58	69.28	78.84	86.96		97.68	42.86
Mont	11	65.22	67.15	70.43	68.41	65.80	61.05	69.48	78.20	86.92	97.68		42.09
SuCMoV_Common_Strain	12	43.88	43.88	43.62	43.11	43.11	37.24	43.11	38.78	44.64	42.86	42.09	

Figure 2.5 Nucleotide sequence similarity of the non-recombinant segment (Fragment 1, 342-bp) at position 1-342 of JVW-186 sequence alignment in conjunction with 10 PVY parental isolates using CLC Main Workbench 6 software.

		1	2	3	4	5	6	7	8	9	10	11	12
JVW-186	1		91.80	86.72	88.57	89.75	82.71	82.03	75.78	76.07	75.68	75.78	53.05
PRI-509	2	91.80		89.75	91.21	92.77	84.18	83.79	75.20	75.68	74.90	74.90	52.27
Adgen	3	86.72	89.75		86.13	87.70	83.40	83.50	76.07	76.37	75.10	75.10	52.37
LYE84.2	4	88.57	91.21	86.13		89.16	83.50	83.30	75.59	76.17	76.17	76.37	53.44
SON41	5	89.75	92.77	87.70	89.16		83.11	83.20	75.88	76.37	75.59	75.49	52.08
SASA-110	6	82.71	84.18	83.40	83.50	83.11		95.41	74.22	74.32	74.80	74.90	52.95
SCRI-O	7	82.03	83.79	83.50	83.30	83.20	95.41		74.02	74.02	74.41	74.51	52.47
SASA-61	8	75.78	75.20	76.07	75.59	75.88	74.22	74.02		98.93	91.02	91.11	53.34
RRA-1	9	76.07	75.68	76.37	76.17	76.37	74.32	74.02	98.93		91.50	91.60	52.86
CH-605	10	75.68	74.90	75.10	76.17	75.59	74.80	74.41	91.02	91.50		99.51	52.95
Mont	11	75.78	74.90	75.10	76.37	75.49	74.90	74.51	91.11	91.60	99.51		53.15
SuCMoV_Common_Strain	12	53.05	52.27	52.37	53.44	52.08	52.95	52.47	53.34	52.86	52.95	53.15	

Figure 2.6 Nucleotide sequence similarity of the recombination event at position 343 (Fragment 2, 1021-bp) of the JVW-186 sequence alignment in conjunction with 10 parental PVY isolates using CLC Main Workbench 6 software.

The 7092-bp non-recombinant segment from position 1365-9307 constitutes a major part of the JVW-186 sequence alignment which extends from the HC-Pro to the CP coding region. A high level of nucleotide sequence similarity (> 80%) to all PVY strain groups (Fig. 2.7) confirms the identity of this isolate.

		1	2	3	4	5	6	7	8	9	10	11	12
JVW-186	1		90.16	89.55	88.57	88.95	88.02	87.83	83.53	83.66	83.76	83.75	70.19
PRI-509	2	90.16		93.23	93.94	94.94	90.79	90.73	84.52	84.63	84.61	84.64	69.71
Adgen	3	89.55	93.23		90.44	91.25	90.86	90.72	85.12	85.22	85.38	85.33	69.92
LYE84.2	4	88.57	93.94	90.44		91.95	88.84	88.74	84.01	84.22	84.23	84.25	69.91
SON41	5	88.95	94.94	91.25	91.95		89.28	89.34	83.81	83.89	83.82	83.85	69.66
SASA-110	6	88.02	90.79	90.86	88.84	89.28		97.23	83.82	83.98	84.15	84.14	69.72
SCRI-O	7	87.83	90.73	90.72	88.74	89.34	97.23		83.88	83.98	84.14	84.10	69.91
SASA-61	8	83.53	84.52	85.12	84.01	83.81	83.82	83.88		99.26	97.35	97.12	70.14
RRA-1	9	83.66	84.63	85.22	84.22	83.89	83.98	83.98	99.26		97.30	97.04	70.15
CH-605	10	83.76	84.61	85.38	84.23	83.82	84.15	84.14	97.35	97.30		99.32	70.29
Mont	11	83.75	84.64	85.33	84.25	83.85	84.14	84.10	97.12	97.04	99.32		70.35
SuCMoV_Common_Strain	12	70.19	69.71	69.92	69.91	69.66	69.72	69.91	70.14	70.15	70.29	70.35	

Figure 2.7 Nucleotide sequence similarity of the a large central non-recombinant segment at position 1365-9307 (Fragment 3, 7942 bp) of the JVW-186 sequence alignment in conjunction with 10 PVY parental strains using CLC Main Workbench 6 software.

The second recombination event at position 9308-9700 is a 392-bp fragment with a high level of nucleotide sequence similarity (98.89%) to the PVY^{O} parental isolate SASA-110 (Fig. 2.8) which confirms the results from RDP4 analysis (Fig. 2.4). This recombination event occurred at the CP region of the JVW-186 sequence alignment.

		1	2	3	4	5	6	7	8	9	10	11	12
JVW-186	1		94.18	92.52	94.18	94.46	87.53	87.26	98.89	98.34	86.98	85.60	60.43
PRI-509	2	94.18		95.29	98.34	97.78	86.98	86.70	93.63	94.18	85.87	84.49	59.89
Adgen	3	92.52	95.29		94.74	94.74	87.26	86.98	91.97	92.52	86.15	84.76	59.35
SON41	4	94.18	98.34	94.74		98.06	87.53	87.26	93.63	94.18	86.98	85.60	59.89
LYE84.2	5	94.46	97.78	94.74	98.06		87.26	87.26	93.91	94.46	85.87	84.49	59.89
Mont	6	87.53	86.98	87.26	87.53	87.26		99.44	86.70	87.26	98.32	96.93	60.22
CH-605	7	87.26	86.70	86.98	87.26	87.26	99.44		86.43	86.98	98.60	97.21	60.49
SASA-110	8	98.89	93.63	91.97	93.63	93.91	86.70	86.43		98.89	86.15	84.76	60.16
SCRI-O	9	98.34	94.18	92.52	94.18	94.46	87.26	86.98	98.89		86.70	85.32	60.43
SASA-61	10	86.98	85.87	86.15	86.98	85.87	98.32	98.60	86.15	86.70		98.04	59.95
RRA-1	11	85.60	84.49	84.76	85.60	84.49	96.93	97.21	84.76	85.32	98.04		59.40
SuCMoV_Common_Strain	12	60.43	59.89	59.35	59.89	59.89	60.22	60.49	60.16	60.43	59.95	59.40	

Figure 2.8 Nucleotide sequence similarity of the recombination event at position 9308 (Fragment 4, 392-bp) of the JVW-186 aligned sequence in conjunction with 10 parental PVY isolates using CLC Main Workbench 6 software.

2.3.6 Phylogenetic analysis

The 342 bp non-recombinant fragment at the 5' end of the sequence alignment for the isolate of PVY (JVW-186) used in this study clustered with PVY^{C} parental isolates (Fig. 2.9). SON41 was isolated from the weed (*Solanum nigrum*) and is prevalent on pepper; LYE84.2 was isolated from tomato and is known to infect other *Solanum* spp. (Moury *et al*., 2002). These isolates belong to the non-potato strains which are a sub-group (C1) of PVY^{C}. PRI-509 was isolated from potato but is more closely related to non-potato strains (C1) based on phylogenetic studies of its coat protein sequence (Dullemans *et al*., 2011). Adgen is a potato strain that belongs to the sub-group C2 which does not infect pepper (Dullemans *et al*., 2011).

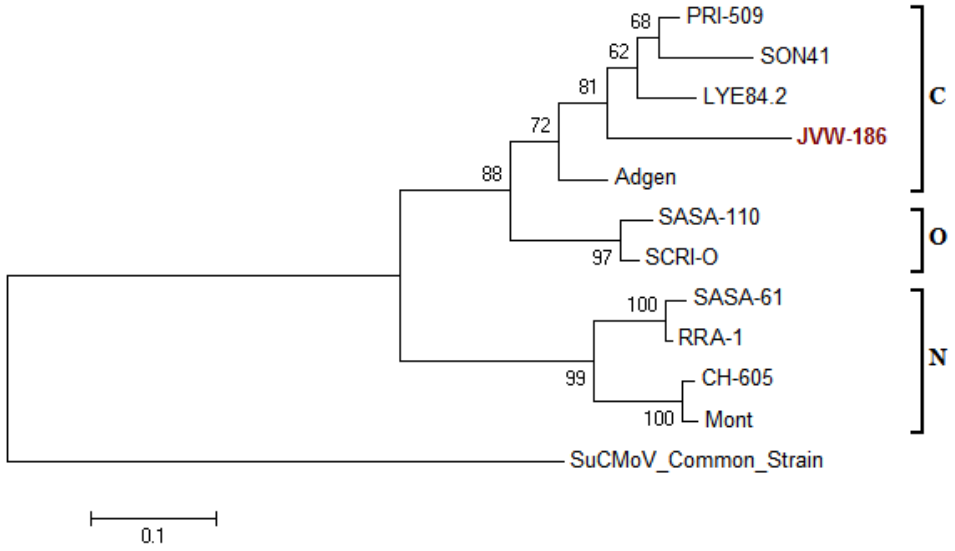

Figure 2.9 Phylogenetic tree constructed from non-recombinant segment (Fragment 1, 342 bp) at position 1-342 of JVW-186 sequence alignment in conjunction with 10 PVY parental isolates using MEGA 5. Bootstrap analysis was performed using 1000 bootstrap samples.

The first recombination event at position 343-1364 (1021 bp) of JVW-186 clustered with PVY^C parental isolates. Although the recombination event in Figure 2.3 shows that the 1021 bp segment is very similar to the PVY^C parental isolate SON41, phylogenetic analysis in Figure 2.10 shows that it is more closely related to LYE84.2. The bootstrap value (88) indicates that the recombinant junction is indistinguishable from other PVY^C parental isolates.

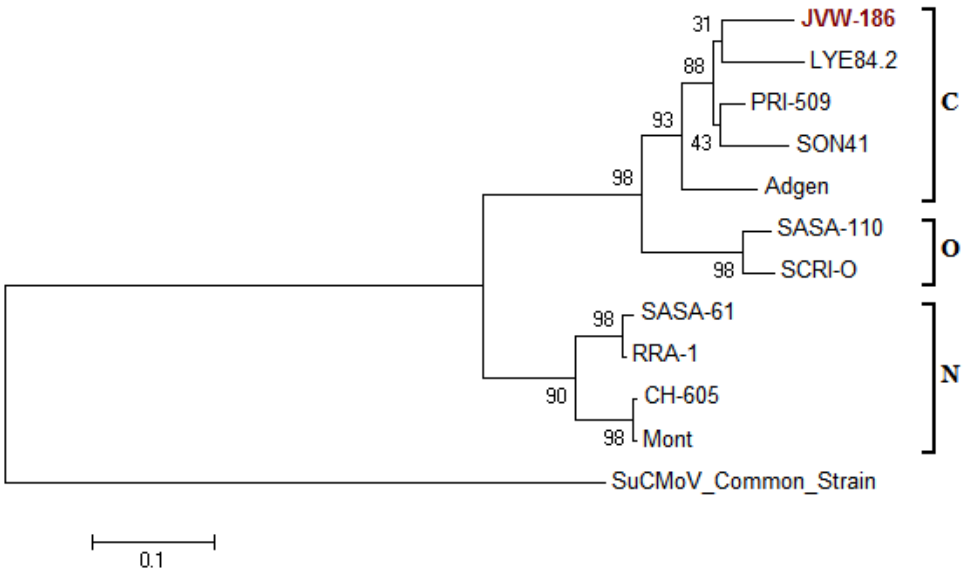

Figure 2.10 Phylogenetic tree constructed from the recombination event at position 343 (Fragment 2, 1021 bp) of the JVW-186 sequence alignment in conjunction with 10 parental PVY isolates. Bootstrap analysis was performed using 1000 bootstrap samples.

The non-recombinant major central domain at position 1365-9307 of JVW-186 did not cluster with any of the parental PVY isolates (Fig 2.11). Phylogenetic analysis of the 7942 bp sequence alignment validates the highly variable nature of PVY. Tree topology (Fig 2.11) indicates the possibility of an ancestral PVY isolate. The bootstrap percentage value provided evidence that the isolate used in this study is indeed PVY.

The second recombination event at position 9308-9700 of the JVW-186 sequence alignment clustered with PVY^{O} parental isolates. Phylogenetic analysis of the recombinant 392 bp fragment (Fig. 2.12) coupled with a relatively high bootstrap percentage value shows that this segment of the alignment is closely related to the PVY^{O} isolate SASA-110 which confirmed the results of RDP4 (Fig. 2.4).

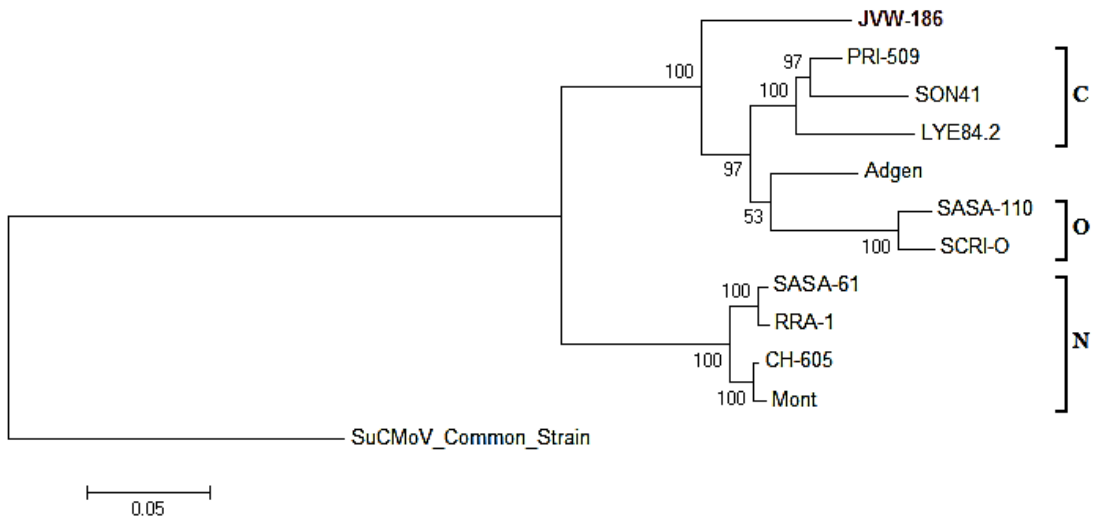

Figure 2.11 Phylogenetic tree constructed from a large central non-recombinant segment at position 1365-9307 (Fragment 3, 7942 bp) of the JVW-186 sequence alignment in conjunction with 10 PVY parental strains using MEGA 5. Bootstrap analysis was performed using 1000 bootstrap samples.

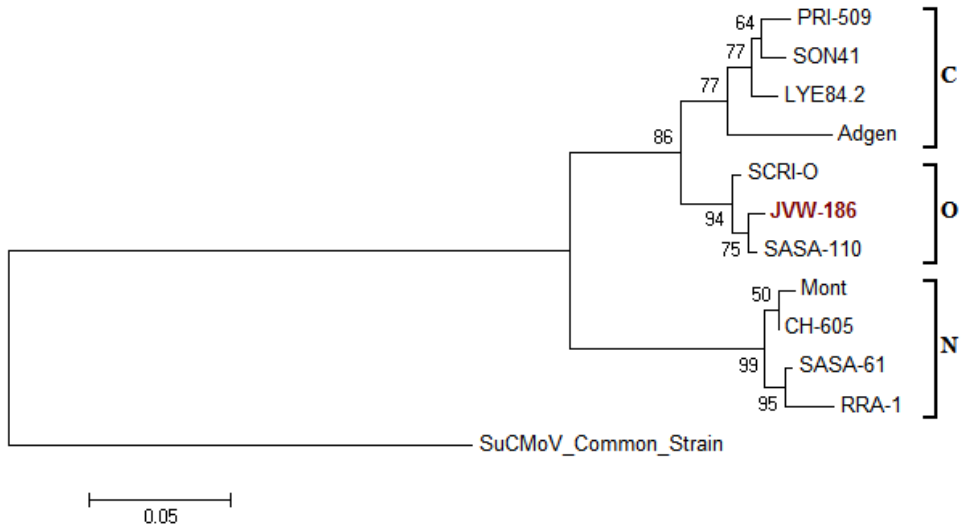

Figure 2.12 Phylogenetic tree constructed from the recombination event at position 9308 (Fragment 4, 392 bp) of the JVW-186 aligned sequence in conjunction with 10 parental PVY isolates using MEGA 5. Bootstrap analysis was performed using 1000 bootstrap samples.

2.4 Discussion

The number of scientific publications devoted to the differentiation of PVY strains far exceeds that of any other virus; however none of published protocols are able to distinguish among all the strains and variants of the virus (Gray *et al*., 2013). In this study the genome of an isolate of PVY infecting pepper in KZN, RSA was sequenced and elucidated. This is the first report of a complete genome sequence of a pepper-infecting PVY isolate from RSA. Numerous PVY strains infecting pepper have been reported from many parts of the world, some of which lack the ability to infect potato and vice versa (Fereres *et al*., 1993).

Although variation exists in the genomic composition of pepper and potato isolates of PVY, monoclonal antibodies can be used to differentiate certain strains of pepper from potato infecting isolates (Soto *et al*., 1994). JVW-186 was positively identified using double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) with PVY^{O}-type antibodies (ADGEN Phytodiagnostics, Europe) which indicated that this isolate belongs to the PVY-O group (data not shown). RDP4 and phylogenetic analysis provided evidence that JVW-186 is a recombinant strain (Fig. 2.4; Fig. 2.9-2.12) and the results from the ELISA test were therefore misleading.

The complete sequence alignment of the PVY JVW-186 isolate in conjunction with 10 parental strains comprised 9700 bp with two recombinant breakpoints at position 343 and 9608 (Fig. 2.4). The second recombination event at position 9608-9700 showed that this segment is similar to the PVY^{O} parental strain SASA-110 (Fig. 2.4) which is in the region of the coat protein gene of the PVY genome. This explains the DAS-ELISA reaction with the PVY^{O} specific monoclonal antibodies. Phylogenetic analysis of the recombinant 392 bp fragment clustered with isolates belonging to the O clade and shared close relations with the parental isolate SASA-110 (Fig. 2.12) which confirmed the results obtained from RDP4 analysis and DAS-ELISA.

A recent field survey (Ibaba and Gubba, 2011) identified a high incidence of PVY in the KZN province in which PVY isolates from pepper reacted positively with PVY^{O}-specific antibodies. JVW-186 used in this study is an isolate collected during this survey. The close proximity of farms from which the virus isolates were collected suggests that these

isolates are similar. Diversity within PVY populations maybe reduced as a result of genetic drift which results from the random effects of bottlenecks (Ayme *et al*., 2006). Furthermore, the vector *Myzus persicae* may amplify the effect of genetic drift during transmission of PVY. Consequently, the least common genetic entities within virus populations are suppressed which may explain the prevalent pathotype infecting pepper in KZN.

Phylogenetic analysis of the non-recombinant 342 bp segment at the 5' end of the sequence alignment clustered with PVY isolated belonging to the C clade (Fig. 2.9). Although PVY JVW-186 clustered within the C clade, the branches indicate that it was not closely related to the parental PVY^{C} strains. Coat protein cistron and whole genome sequence alignment data distinguishes C isolates from PVY^{N} and PVY^{O} strains (Dullemans *et al*., 2011). Pepper-infecting isolates of PVY that are mechanically inoculated onto potato plants do not cause systemic infection; similarly, pepper plants are not systemically infected with PVY^{N} strains (Gebre-Selassie *et al*., 1985; McDonald and Kristjansson, 1993; d'Aquino *et al*., 1995; Valkonen *et al*., 1996). Some potato infecting strains belonging to the O and C groups are able to infect pepper (Blanco-Urgoiti *et al*., 1998). Hence, the possibility arises that JVW-186 with spliced O and C type fragments may have originated from potato. Further tests are required to assess for symptom development in potato crops inoculated with this isolate.

Nineteen tomato cultivars mechanically inoculated with the isolate JVW-186 were systemically infected (data not shown) and severe mottling and mosaic symptoms were observed in the propagation host *N*. *tabacum* cv. Xanthi. According to Stobbs *et al* (1994) and Blancard (1998), the majority of PVY isolates that originate from pepper and potato are able to infect tobacco and tomato cultivars that lack the necessary resistance. This data suggests that the evolution PVY strains are influenced by selective host species such as those from potato and pepper.

Phylogenetic analysis of the large non recombinant 7942 bp segment showed that it did not cluster with PVY isolates from any of the clades (Fig. 2.11). The unique 7942 bp segment suggests the possibility of a novel strain of PVY that has evolved due to selection pressure. Furthermore, the likelihood of an ancestral PVY isolate based on

the elucidation of the genome is questionable. According to sequence data analysis using RDP4 (Fig. 2.4) in combination with phylogenetic analysis (Fig. 2.11), regions of the complete genome sequence of this isolate may belong to a completely different strain group of PVY. This high level of sequence similarity of individual non-recombinant and recombinant segments of the sequence alignment shared among the various PVY groups (Fig. 2.5 - 2.8), together with the identification of typical potyvirus-like virions using transmission electron microscopy and a 30-kDa coat protein from the SDS-PAGE protein assay (Fig. 2.2) confirms that JVW-186 is indeed an isolate of PVY and the possibility of a novel recombinant strain. In addition, two ORF's identified at position 186 and 2915 of the sequence alignment is the site of polyprotein synthesis and the frameshift translation protein P3N-PIPO respectively. The identification of these ORF's in conjunction with ten functional proteins at various coding regions across the JVY-186 sequence alignment is typically associated with potyviruses. Furthermore, molecular analysis of the amino acid residues showed numerous conserved motifs at specific positions in the gene encoded products of JVW-186. In JVW-186 Ile was substituted with Met in the highly conserved KITC motif which together with the PTK motif is responsible for aphid transmission (Atreya *et al*., 1992; Blanc *et al*., 1998). According to Sasaya *et al* (2000), a substitution from Lys to Glu in the KITC tetrapeptide resulting in EITC is associated with non-aphid transmissible strains such as PVY^{C}. PVY^{C} strain SON41 shares a similar amino acid substitution in KITC motif as JVW-186 resulting in KMTC. Since SON41 is aphid transmissible (Moury *et al*., 2007), it suggests that this amino acid change does not abolish aphid transmission. Moreover it provides evidence that JVW-186 has been widely distributed throughout the KZN province as a result of aphid transmission.

JVW-186 was identified as pathotype 0 based on protein analysis of the VPg which is a virulence determinant toward PVY recessive resistance alleles at the *pvr2-elF4E* locus in pepper crops (Fig. 2.3). Furthermore, its ability to overcome this allelic series of recessive genes ($pvr2^{+}$, $pvr2^{1}$ and $pvr2^{2}$) at the *pvr2-elF4E* resistance locus in pepper cultivars can be used as criteria to confirm the pathotype of this isolate. Subsequent control methods can be implemented based on our understanding of the genetic

mechanisms in the host that are able to resist virus infection; as well as the ability of the infecting pathotype to overcome resistance.

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Chapter 3

Screening for resistance to an isolate of PVY infecting pepper (*Capsicum annuum* L.) in KwaZulu-Natal (KZN), Republic of South Africa

Abstract

Potyvirus resistance is a recessive trait which comprises more than one third of all known virus recessive resistances. Two recessive alleles (*pvr2*^{1} and *pvr2*^{2}) located at the *pvr2* resistance locus in pepper (*Capsicum annuum* L.) confer pathotype mediated resistance to potato virus Y (PVY). PVY is an economically important virus of field grown solanaceous crops throughout the world. In the province of KwaZulu-Natal (KZN), Republic of South Africa (RSA), the incidence of PVY infection has been reported to significantly reduce the yield and quality of cultivated peppers. This study aims to evaluate commercially grown pepper lines for resistance against PVY. Six F_1 pepper lines were challenged with the isolate of PVY previously propagated onto *Nicotiana tabacum* cv. Xanthi leaf material using mechanical inoculation under greenhouse conditions. Each line was assessed for resistance to PVY by visual screening for disease severity and quantitative triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) for virus load. The pepper lines were further characterized using tetra primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR) to identify and differentiate the presence of homozygous/heterozygous resistance alleles at the *pvr2* locus that confer PVY resistance. Evaluations revealed varying levels of resistance and susceptibility in pepper lines challenged with PVY. The most susceptible pepper line was Benno, although high levels of susceptibility were observed in IP, Excellence and Mantenga. Double Up and Cecelia remained symptomless throughout the study. The *pvr2*$^{+}$ allele was positively identified in all the susceptible pepper lines which confirm that the presence of this allele is dominant for PVY susceptibility. The information generated in

this study can be incorporated into breeding programs intended to control PVY on pepper in KZN.

3.1 Introduction

Potato virus Y (PVY) is the type member of the genus *Potyvirus* which belongs to the family Potyviridae (Aramburu *et al*., 2006). It is transmitted by several aphid species in a non-persistent manner causing extensive damage to many economically important crops such as pepper (*Capsicum annuum* L.), tomato (*Solanum lycopersicum* Mill.), tobacco (*Nicotiana* spp.) and potato (*Solanum tuberosum*) (De Bokx and Huttinga, 1981). The virus has flexuous rod shaped particles comprising a single-stranded, positive-sense RNA molecule of approximately 9.8 kb in length, with a genome-linked viral protein (VPg) covalently attached at the 5' end and a poly (A) tail at the 3' end (Shukla *et al*., 1994). The single large polyprotein encoded by the viral RNA is cleaved by three viral encode proteases, producing nine functional genes (Dougherty and Carrington, 1988).

In KwaZulu-Natal (KZN), Republic of South Africa (RSA), the prevalence of PVY has been reported to cause yield losses of up to 50% in areas where peppers are cultivated (Budnik *et al*., 1996; Ibaba and Gubba, 2011; Trench *et al.*, 1992). The methods currently used to mitigate the detrimental impact of PVY have been met with limited success. However, the use of resistant varieties (where available) is the best alternative to manage PVY on pepper. By screening various cultivars of pepper for resistance to PVY, genotypes expressing high levels of resistance can be incorporated into breeding programs that may confer durable, natural and safe methods of resistance.

Recently, much effort has gone towards a better understanding of the molecular mechanisms associated with genes that confer natural resistance (Maule *et al*., 2007). These advancements have led to the molecular characterization of dominant and recessively inherited traits and their function in gene silencing (Maule *et al*., 2007). A well-known active defense mechanism which leads to the formation of a visible necrotic

lesion is the so-called hypersensitive response (HR) during which the movement of the virus is restricted as a result of programmed cell death (Goldbach *et al*., 2003). This defense mechanism is triggered upon the recognition of the virus during which dominant resistant genes (R genes) are matched with avirulence genes in the virus (Gururani *et al*., 2012). Most natural resistance genes are monogenic dominant R genes which are predominant against bacteria and fungi; virus resistance on the other hand is largely a result of recessive genes (Robaglia and Caranta, 2006).

Resistance to potyviruses is a recessive trait which constitutes 40% of all known recessive resistance virus genes (Provvidenti and Hampton, 1992). The genetic resources of pepper that confer resistance to PVY fall into this category of resistance (Ruffel *et al*., 2002). The *pvr2*^{1} and *pvr2*^{2} recessive alleles located at the *pvr2* resistance locus in pepper mediate pathotype specific resistance (Kyle and Palloix, 1997; Ruffel *et al*., 2002). Pathoypes of PVY that infect pepper have been classified as 0, 1 and 1-2 based on their ability to overcome the recessive resistance alleles (*pvr2*$^{+}$, *prv2*^{1} and *pvr2*^{2}) at the *prv2* locus respectively (Gebre-Selassie *et al.*, 1985). The *pvr2*^{1} allele provides effective resistance against PVY pathotype 0 and the *pvr2*^{2} allele is effective against pathotypes 0 and 1 however, both genes are overcome by the PVY pathotype 1-2 (Ruffel *et al*., 2002). Pathotype 0 and 1 constitute the vast majority of pepper isolates collected during field surveys throughout the world (Palloix *et al*., 1994). Additionally, pathotype 1-2 can be selectively acquired by means of serial inoculations on pepper plants carrying the *pvr2*^{2} allele (Gebre Selassie *et al*., 1985). Serological studies indicate that isolates of PVY infecting pepper are closely related to the PVY^{O} strain group; however no relationship has been established between serotypes and pathotypes (Soto *et al*., 1994).

Numerous studies have implicated the *eIF4e* translation initiation factor in potyvirus mediated resistance (Maule *et al*., 2007; Robaglia and Caranta, 2006; Ruffel *et al*., 2002). Molecular analysis of the *pvr2* gene in pepper established a link to the *eIF4E* eukaryotic translation initiation factor (Ruffel *et al*., 2005). Interestingly, sequence data of *eIF4E* genes from PVY-susceptible pepper crops differed to that of PVY-resistant peppers by two amino acid changes (Ruffel *et al*., 2002). The *eIF4E* is a key

component of protein synthesis during initial infection and is further required for efficient translation of mRNAs (Kawaguchi and Bailey-Serres, 2002). It is proposed that mRNA translation takes place in a closed-loop orientation during which the 5'- and 3'- termini are brought together as they interact with translation initiation factors (Kawaguchi and Bailey-Serres, 2002). Evidently, the genomic and sub-genomic mRNAs of positive-sense RNA plant viruses seldom share a similar structure with host mRNAs (5' cap and a poly adenylated tail) and frequently lacks one or both structures (Fauquet *et al*., 2005). PVY has a polyadenylated tail at the 3'- end but instead of a 5'- cap structure they have a covalently attached VPg at the 5'-end (Nieto *et al*., 2006).

Non-synonymous mutations at specific regions in the VPg cistron of PVY determine virulence toward recessive resistance alleles at the *pvr2-eIF4E* locus in pepper (Moury *et al*., 2004; Ayme *et al*., 2006). Successful virus infection is determined by the interaction between the VPg and *eIF4E* (mRNA 5' cap binding protein) (Grzela *et al*., 2006). According to Borgstrom and Johansen (2001), amino acid mutations in the VPg or *eIF4E* hamper the interaction between these proteins and are the basis of natural recessive resistance to potyviruses. The use of molecular methods to identify the type of recessive alleles that control PVY resistance in pepper is a step closer to understanding the mechanisms of infection. Amplification mutation system polymerase chain reaction (ARMS-PCR) has been primarily used to screen for mutations in diagnostic procedures of human and veterinary science (Piccioli *et al*., 2006); and is an emerging technique in plant studies able to distinguish alleles in both homozygous and heterozygous genotypes. Rubio *et al* (2008) designed primer combinations based on single nucleotide polymorphisms (SNPs) able to detect and differentiate alleles ($pvr2^{+}$, $pvr2^{1}$, $pvr2^{2}$ and $pvr2^{3}$) at the *pvr2-eIF4E* locus in pepper using the ARMS-PCR procedure. These functional markers provide confirmation of genetic resources that are frequently used in breeding programs.

In view of the various issues surrounding the current methods to manage PVY on pepper, identifying sources of resistance can ultimately mitigate the effects of this disease in commercial and small-scale farms. Furthermore, genetic resources from resistant pepper crops can be characterized and introgressed into commercial varieties.

Against this background, F_1 sweet pepper lines were evaluated for resistance to PVY using a disease rating scale and ARMS-PCR.

3.2 Materials and Method

3.2.1 Test lines

Six commercially important pepper lines were used in this study. These are F_1 hybrids that are grown in KZN by small-scale farmers sold by seed companies. Fifteen seeds from each pepper line were sown in seedling mix containing finely milled pine bark and maintained in a glasshouse facility at the University of KwaZulu-Natal (UKZN). A list of the various F_1 lines provided by Sakata and Pro Seed are summarized in Table 3.1.

Table 3.1 Six sweet pepper lines evaluated for resistance against PVY

Pepper Line	Seed Company
Benno	Sakata
IP	Sakata
Mantenga	Pro Seed
Double Up	Sakata
Excellence	Sakata
Cecelia	Sakata

Mantenga was used as the positive susceptible control. Three weeks post germination; twelve seedlings per line were individually transplanted into 150 mm pots filled with potting mix containing pine bark provided by UKZN. Nine plants per line were mechanically inoculated with PVY and three were used as controls (Section 3.2.2). The pepper plants were then grown and fertilized in an experimental glasshouse facility at approximately 26°C.

3.2.2 Mechanical inoculation of test lines

Virus inoculum was prepared from freshly harvested tobacco (*Nicotiana tabacum* cv. Xanthi) leaves previously infected with a pepper isolate of PVY which had been purified using single-lesion isolation from *Chenopodium amaranticolor*. Inoculum consisted of infected tobacco tissue homogenized in ice-cold 50 mM sodium-phosphate buffer, (pH 7.4) (1 g of leaf tissue: 5 ml buffer). Prior to inoculation (at the four leaf stage of development), leaves of test plants were lightly dusted with 400 mesh carborundum and manually rubbed with PVY inoculum. Control plants were mock inoculated with sterile phosphate buffer. The inoculum was replaced every 10 min to ensure that the virus remained viable throughout the procedure. The leaves were rinsed with ice-cold distilled water to remove excess inoculum. The inoculation procedure was repeated after seven days. Inoculated plants were maintained in an insect free glass house (26°C) and observed for virus symptom development over a four week period.

3.2.3 Screening for resistance to PVY

3.2.3.1 Visual screening

Fourteen days post-inoculation; pepper lines were visually assessed for symptom severity according to a five point rating scale proposed by Canady *et al* (2001). The rating scale: 1 = no visible symptoms, 2 = mild chlorosis and limited leaf distortion, 3 = moderate chlorosis, leaf distortion with some plant stunting, 4 = severe chlorosis, leaf distortion and plant stunting, 5 = severe chlorosis, leaf distortion and extreme stunting. Plants were rated once a week over a period of eight weeks.

3.2.1.2 Virus accumulation in host tissue

The accumulation of PVY virions in host tissue was quantified by means of triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) using Adgen (Europe) phytodiagnostic ELISA kits. Approximately 1 g of plant tissue was collected from the actively growing parts of the individual plants. The leaf material collected from each plant was individually weighed and transferred to 2 ml microcentifuge tube containing four to five plastic beads. Each microcentifuge tube was loaded with 1 ml of extraction buffer [PBS-Tween 20, 2% (w/v) PVP-40 polyvinyl pyrrolidone pH 7.4] and

Table 3.2 Allele specific amplification of recessive genes in pepper using tetra primer ARMS-PCR (Rubio *et al*., 2008).

Primer	Sequence	Expected Size (bp)	Allele	Annealing Tm (°C)
A614G				
Forward Inner Primer	AATGGAAGCAGTTTCTGGATTACAGAGG	177	2^3	56
Reverse Inner Primer	ATACGTGAAATATGAAGCCAACACGGT	127	2^+, 2^1, 2^2	56
Forward Outer Primer	CGTTGTAACTATTCTTACCGCCATGCTT	249		56
Reverse Outer Primer	TGTAACGATTCTTTGCATTTCTGTCGA	249		56
G325A				
Forward Inner Primer	CACCCAAGCAAGTTAGTTGTGGGAGAAA	198	2^2	48
Reverse Inner Primer	AATTTTATGCTTGAAACAATGTAATTC	288	2^+, 2^1, 2^3	48
Forward Outer Primer	GTACTTATGTGAATTTGGTGTCTGCCTT	431		48
Reverse Outer Primer	TACTAGAGTGACCAATCACTACGAGCTG	431		48
T200A				
Forward Inner Primer	TCATGGACTTTCTGGTTTGATAATCCGGT	199	2^+	54
Reverse Inner Primer	CCAAGCAGCTTGTTTCGATTTCGTCT	258	2^1, 2^2, 2^3	54
Forward Outer Primer	TCCCGAAAGTAAAAAAAGCACACAGCAC	402		54
Reverse Outer Primer	TCGTGATTGTTCGATTCCCCTAATACCC	402		54

placed in a bead-beater for 1 min to homogenize the samples. The homogenate was then centrifuged for 5 min at 13000 x *g* at room temperature. The supernatant was transferred to the respective wells of high-binding ELISA plates for quantification. After adding the substrate, samples were incubated at 37°C for 1 h in the absence of light. The absorbance values measured at A_{405}nm were read using an Anthos 2001 microtiter plate reader. The positive control was provided and used according to the instructions of the manufacturer (Adgen Europe Ltd). The sap from mock inoculated disease free, control plants was used as the negative controls. Each sample was loaded in duplicate wells and the average absorbance value for the duplicate wells of nine plants per line were analysed statistically using Genstat (Payne *et al*., 2011). Samples that indicated absorbance readings greater than two-fold the value of the negative control were regarded as positive for the presence of PVY.

3.2.4 DNA extraction and genotyping of pepper lines

DNA was extracted according to the method described by Edwards *et al* (1991) with the following amendments; leaf tissue was homogenized in a microcentrifuge tube containing five to six plastic beads and 400 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS) using a bead beater for 30 s. The homogenate was then placed on a heating block at 65°C for 10 min and centrifuged at 16,000 x *g* for 3 min. An equal volume of ice-cold isopropanol was added to the supernatant in a new microcentrifuge tube and the contents were mixed by pipetting. Samples were incubated at -20°C for 30 min and centrifuged at 16,000 x *g* for 5 min. The pellets were washed in 70% ethanol, air-dried for 15 min and re-suspended in 50 µl of sterile TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) buffer.

Tetra-primer ARMS-PCR was used to assay the recessive alleles at the *pvr2-eIF4E* locus in pepper genotypes according to Rubio *et al* (2008). Recessive alleles were differentiated using three sets of tetra primers (A614G, G325A and T200A) that are able to identify SNP's at this locus. The primer sequences, annealing temperatures and expected band sizes are summarized in Table 3.2. ARMS-PCR was carried out in 20 µl reactions consisting of 10 µl KAPA 2G Fast DNA polymerase (Kapa Biosystems, Lasec, South Africa), 200 nM of each primer and 30 ng of genomic DNA. ARMS-PCR was

performed at 95°C for 3 min; 35 cycles of 15 s at 95°C, 20 s at the annealing temperature specified for each set of primers listed in Table 3.2, 20 s at 72°C and a final elongation for 2 min at 72°C.

3.3 Results

3.3.1 Visual screening for resistance

No symptoms were observed for Double Up and Cecelia. Benno, IP, Mantenga and Excellence expressed severe symptoms (Fig. 3.1). This was expected for Mantenga which was used as the positive control. Benno was found to be the most susceptible line exhibiting severe mottling, stunting and leaf distortion (Fig 3.2).

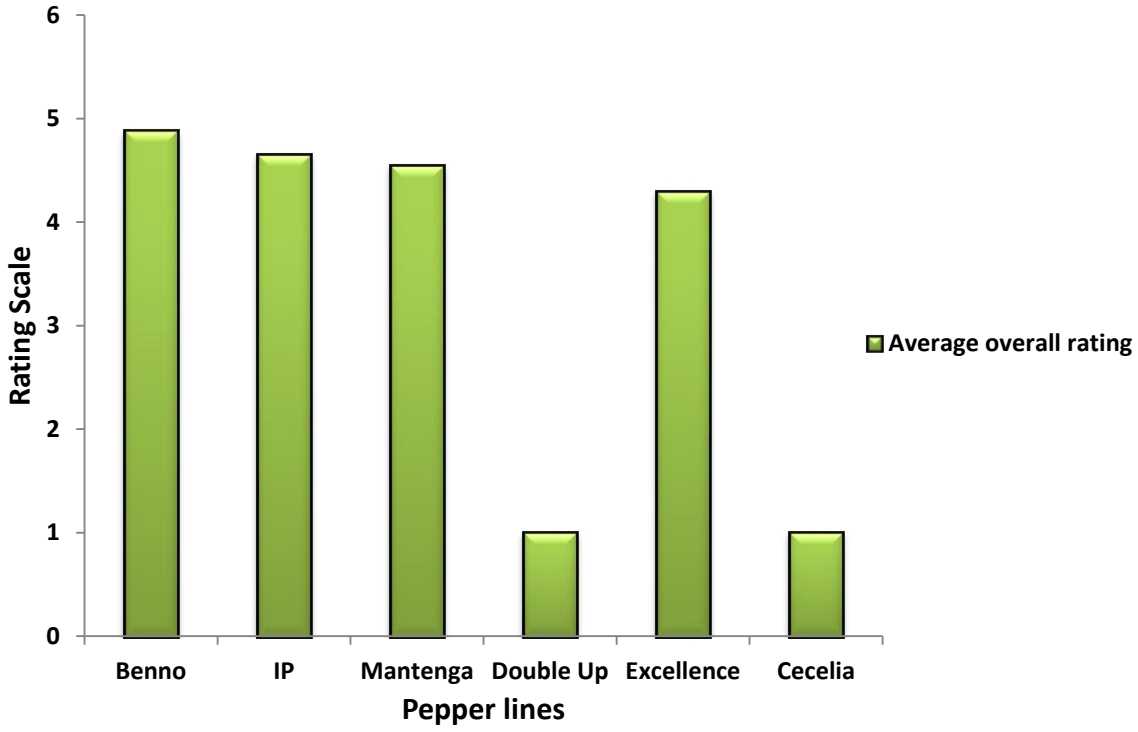

Figure 3.1 Disease severity reactions in the evaluation of six F_1 pepper lines for resistance against PVY.

Figure 3.2 Pepper plants visually screened for resistance to PVY. A: Comparison of an asymptomatic plant (Cecelia) on the left with the positive control (Mantenga) on the right expressing severe mottling, chlorosis and leaf distortion; B: A resistant line (Double Up) on the left showing no symptoms compared with the most susceptible pepper line (Benno) on the right exhibiting severe mottling, stunting, chlorosis and leaf distortion; C: Single leaf isolated from the positive control (Mantenga) showing severe infection.

3.3.2 Quantitative ELISA

Benno had the highest TAS-ELISA absorbance value (Fig 3.3). This value was positively correlated to the symptom expression shown in Figure 3.2B. Similar results were obtained for IP, Mantenga and Excellence which corresponded to the data shown in Figure 3.1. Double Up and Cecelia had extremely low absorbance values that were more or less on par with the values obtained for the negative controls. Although Mantenga and Excellence developed symptoms, the severity was significantly less than those expressed in Benno and IP ($P<0.001$) (Table 3.3). Double Up and Cecelia remained asymptomatic and had significantly lower absorbance values to those obtained in the other four pepper lines used in this study. These results indicate that Double Up and Cecelia are immune to PVY infection. In addition, the immunity maybe linked to genetic resourced that confer PVY resistance.

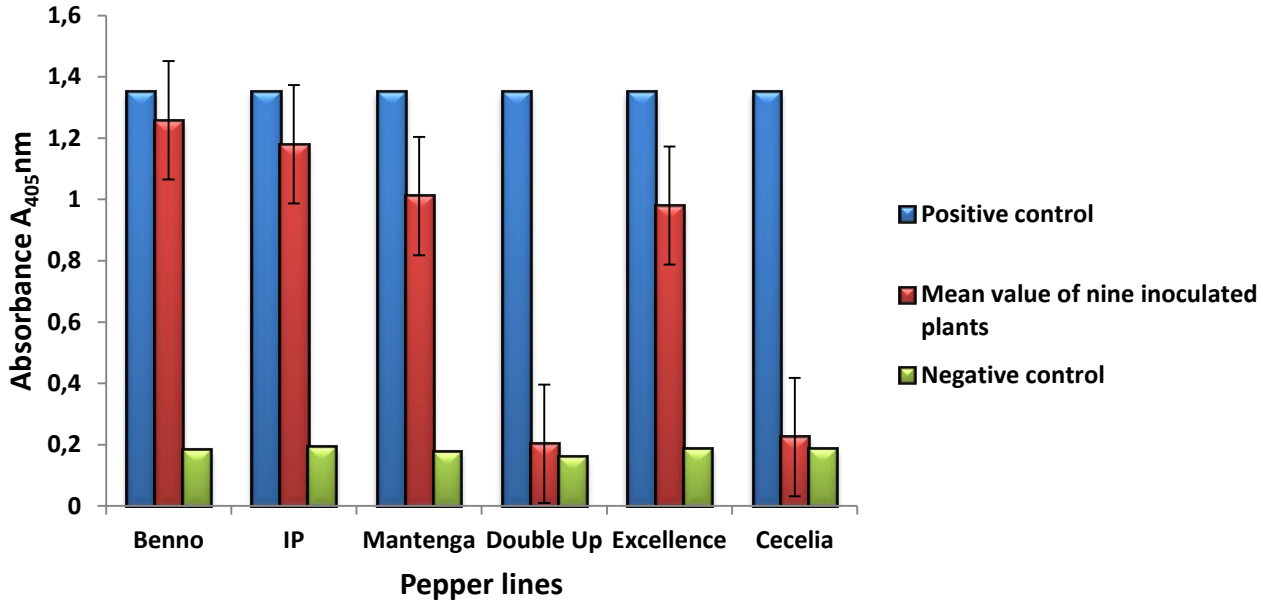

Figure 3.3 Measurements of virion accumulation (A_{405}nm) in the evaluation of six F_1 pepper lines for resistance against PVY using TAS-ELISA. The error bars indicate standard error.

Table 3.3 Quantification of viral load (A_{405}nm) in six F_1 pepper lines mechanically inoculated with PVY.

Pepper Lines	Absorbance	
Double Up	0.2073	a
Cecelia	0.215	a
Excellence	0.9333	b
Mantenga	1.0157	b
IP	1.19	c
Benno	1.2213	c
s.e.d	0.03208	
l.s.d	0.07148	
C.V.%	4.9	
P	<0.001	

3.3.3 Genotyping of pepper lines

The genotype of each pepper line was determined from the band amplification pattern using three sets of tetra primers A614G (Fig. 3.4 A), T200C (Fig. 3.4 B) and G325A (Fig. 3.4 C). The common allele is present in all pepper genotypes and varies in size according to each tetra-primer set. The common alleles in lane 1 and lane 2 (B) were not clearly defined due to competition between the fragments being amplified (Rubio *et al*., 2008) and has no impact on the outcome of this experiment. Since the recessive alleles (*pvr2*$^{+}$, *pvr2*^{1}, *pvr2*^{2} and *pv2*^{3}) at the *pvr2-eIF4E* locus regulate resistance/susceptibility in pepper plants to pathotypes of PVY (Ben Khalifa *et al*., 2009), determining the genotype of each line can be matched with their corresponding phenotype. Pepper crops displaying either a two-band pattern or a three band-pattern including the common amplicon in each gel is associated with homozygous and heterozygous genotypes respectively. The lack of a 177 bp amplicon (Fig. 3.4 A) indicates that *pvr2*^{3} resistance recessive allele was absent in all six F_1 pepper lines. The generation of a 199 bp (*pvr2*$^{+}$) (Fig. 3.4B) was detected in four pepper lines (Benno, IP, Mantega and Excellence). This data shows that the *pvr2*$^{+}$ allele is associated with the development of symptoms and confirmed by the subsequent serology assay of

these pepper genotypes showing the presence of PVY (Fig. 3.1 - Fig. 3.3). Furthermore, only homozygous *pvr2*^{1} or *pvr2*^{2} genotypes Double Up and Cecelia were asymptomatic which indicates that these alleles confer resistance to the isolate of PVY used in this study.

Homozygous/heterozygous recessive alleles for each line were identified using the amplification band patterns (Fig. 3.4, Table 3.4). All pepper lines carrying the *prv2*$^{+}$ allele developed symptoms (Fig. 3.1) and the accumulation of virus particles in host tissue (Fig. 3.3). Cecelia (*pvr2*^{2}/*pvr2*^{2}) and Double Up (*pvr2*^{1}/*pvr2*^{1}) remained asymptomatic throughout the trial (Fig. 3.1). Furthermore the ELISA assay was negative for the detection of PVY virions in these two lines (Fig. 3.3). This indicates that the *pvr2*^{1} and *pvr2*^{2} alleles confer resistance to the isolate of PVY used in this study.

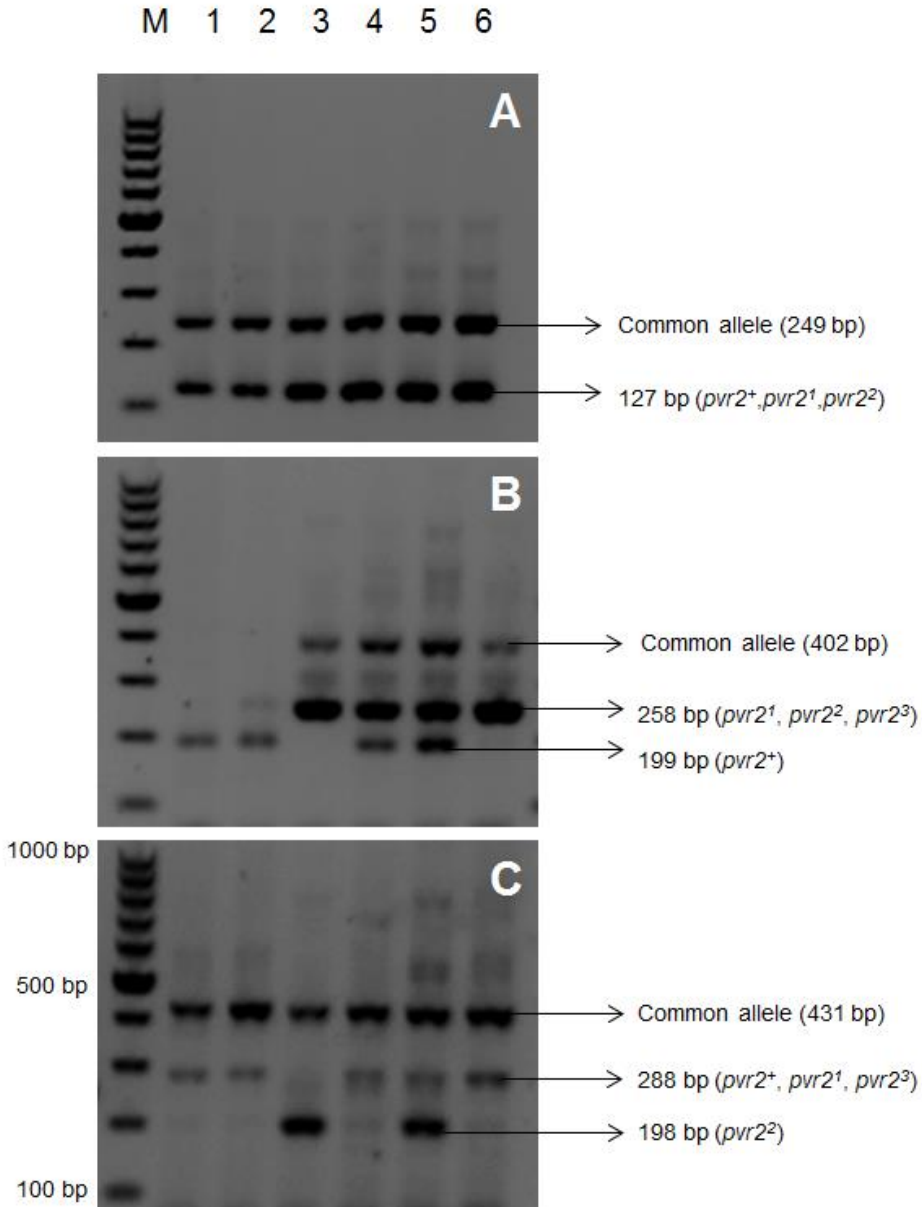

Figure 3.4 Identification and differentiation of homozygous/heterozygous recessive alleles at the *pvr2-eIF4E* resistance locus of six F_1 pepper lines using tetra-primer ARMS-PCR. Lane M: 100 bp molecular weight marker (Fermentas); Lane 1: IP; Lane 2: Mantenga, Lane 3: Cecelia; Lane 4: Excellence; Lane 5: Benno; Lane 6: Double Up.

Table 3.4 Genotyping of pepper lines using the tetra primer ARMS-PCR procedure.

Primer	**A614G**		**T200A**		**G325**		
bp	**127**	**177**	**258**	**199**	**288**	**198**	
pvr	***2*$^{+}$,*2*^{1},*2*^{2}**	***2*^{3}**	***2*^{1},*2*^{2},*2*^{3}**	***2*$^{+}$**	***2*$^{+}$,*2*^{1},*2*^{3}**	***2*^{2}**	
Pepper line							**Genotype**
IP	+	-	-	+	+	-	*pvr2*$^{+}$/*pvr2*$^{+}$
Mantenga	+	-	+	+	+	-	*pvr2*$^{+}$/*pvr2*$^{+}$
Cecelia	+	-	+	-	-	+	*pvr2*^{2}/*pvr2*^{2}
Excellence	+	-	+	+	+	-	*pvr2*^{1}/*pvr2*$^{+}$
Benno	+	-	+	+	+	+	*pvr2*^{2}/*pvr2*$^{+}$
Double Up	+	-	+	-	+	-	*pvr2*^{1}/*pvr2*^{1}

+: positive amplification of allele using a specific set of tetra primers, -: no band observed at the expected band size, (bp) is the expected band size in base pairs.

3.4 Discussion

The evaluation revealed two resistant pepper lines i.e. Cecelia (*pvr2*^{2}/*pvr2*^{2}) and Double Up (*pvr2*^{1}/*pvr2*^{1}). These pepper lines remained symptomless throughout the period of the evaluation. In addition, the absorbance readings (A_{405}) obtained from the apical leaves of these pepper lines provided evidence that PVY virions did not move or accumulate systemically. Mantenga (positive control), IP, Excellence and Benno developed severe mottling, leaf distortion and stunting. The severe nature of symptoms expressed in Benno (Fig. 3.2B) was linked to the high absorbance values obtained (Fig. 3.3). The inability to detect virions using TAS-ELISA (Table 3.3) in asymptomatic leaf tissue indicates that Cecelia and Double Up are operational immune plants as described by Boiteux *et al*., 1996. According to Ponz and Bruening (1986), an

operational immune plant is phenotypically described as being symptomless and restricting the accumulation of virus particles to levels above those introduced during inoculation.

Natural resistance to PVY in pepper is a recessive trait which suggests that Benno and Mantenga did not possess the necessary genetic resources to overcome infection. This type of resistance has been linked to the eukaryotic translation initiation factor *eIF4E* which belongs to a small multigenic family (Ruffel *et al*., 2005). Recessive alleles (*pvr2*^{1} and *pvr2*^{2}) at the *pvr2-eIF4E* resistance locus confer pathotype mediated resistance to PVY in pepper (Kyle and Palloix, 1997; Ruffel *et al*., 2002). Infection and systemic spread is often the result of peppers carrying the *pvr2*$^{+}$ allele which is dominant for susceptibility (Ben Khalifa *et al*., 2009). In addition, these susceptible pepper cultivars may lack the recessive allele required for resistance to the infecting pathotype. Since PVY pathotypes (0, 1, 1-2) are able to overcome the recessive resistance to specific alleles (*pvr2*$^{+}$, *pvr2*^{1} and *pvr2*^{2}) respectively, it is important to identify these genes so that a relationship can be established between the infecting pathotype and pepper cultivar.

Studies conducted by Ruffel *et al*. (2002) showed that the amino acid sequence of the translation initiation factor *eIF4E* obtained from susceptible pepper genotypes differed from resistant genotypes at only two amino acids indicating that slight variations in the sequence are adequate to confer PVY resistance. This indicates that the resistant phenotype (mediated by the locus) is a result of genotype specific substitutions (Ruffel *et al*., 2002). Moreover, in pepper-PVY recessive resistance the virus genome linked protein (VPg) cistron is a virulence determinant toward alleles at the *pvr2* locus (Borgstrom and Johansen 2001; Moury, 2004). According to Masuta *et al* (1999), cell to cell movement of PVY is inhibited by the mechanisms involving the central region of the VPg cistron and recessive resistance alleles in pepper. The VPg interacts with the *eIF4E* and hence, enables successful viral infection (Nieto *et al*., 2006). The genetic mechanisms highlighted above indicate that a physical association between the VPg and *eIF4E* is needed for PVY infection to occur. Furthermore, amino acid mutations may occur in specific regions of the VPg cistron or near the cap of the *eIF4E* protein

which affect this interaction (Borgstrom and Johansen 2001). This loss of host components is the basis of natural recessive resistance to PVY in pepper.

Provvidenti and Hampton, (1992) acknowledged the unusually high percentage of recessively inherited genes that confer resistance to potyviruses (approximately 40%). Therefore it can be suggested that the immune pepper lines obtained in this study, carry the recessive genes that mediate resistance against PVY. Against this background molecular markers were used to identify recessive alleles (*pvr2*$^{+}$, *pvr2*^{1}, *pvr2*^{2} and *pvr2*^{3}) at the *pvr2-eIF4E* resistance locus of all six pepper lines. These functional markers were designed by Rubio *et al* (2008) using the ARMS-PCR procedure. From Table 3.3, all pepper lines carrying the *pvr2*$^{+}$ allele corresponded with the development of symptoms (Fig. 3.1) which confirms that the *pvr2*$^{+}$ allele is dominant for susceptibility to PVY as shown by Ben Khalifa *et al*., 2009. The lines Double Up and Cecelia were genotyped as homozygous lines carrying the respective *pvr2*^{1} and *pvr2*^{2} recessive alleles based on the amplification band patterns (Fig. 3.4) and remained symptomless throughout the evaluation. Furthermore, PVY virions were not detected from leaf tissue samples in serological assays. This suggests that these lines have the necessary genetic resources for PVY mediated resistance. In addition, the isolate of PVY used in this study was unable to overcome the resistance genes *pvr2*^{1} and *pvr2*^{2} identified in Double Up and Cecelia respectively which indicates that the isolate of PVY infecting pepper in KZN belongs to PVY- pathotype 0.

The nature of resistance genes that are suitable for breeding into commercial varieties are dependent upon durability. Mutation occurs frequently among PVY isolates all over the world which explains why most of the currently circulating isolates are a result of recombination (Cuevas *et al*., 2012). The probability of resistance-breaking strains in terms of the evolutionary potential of PVY is high (Cuevas *et al*., 2012); therefore, the identity of the infecting pathotype in relation to the cultivated variety is important when breeding for resistance. Routine surveys to identify the variability of PVY isolates infecting pepper in KZN are required to develop varieties that provide durable resistance. Furthermore, the homozygous sources of resistance identified in pepper

lines Cecelia and Double Up during this study can be used in breeding programs intended to control PVY infecting pepper in KZN.

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Chapter 4

Development of homozygous pepper (*Capsicum annuum* L.) lines carrying potato virus Y (PVY) resistance genes (*pvr2*^{1} and *pvr2*^{2}) using marker-assisted selection (MAS)

Abstract

Pepper (*Capsicum annuum* L.) is an important vegetable crop grown and consumed worldwide. Potato virus Y (PVY) is a highly destructive, globally distributed pathogen which significantly reduces the yield and quality of cultivated peppers. Chemical and cultural methods have proven ineffective in controlling PVY; therefore the development of resistant varieties is the best alternative to manage PVY diseases on pepper. Several alleles at the *pvr2* locus are known to control recessive resistances to PVY in pepper. In this study, homozygous F_2 pepper lines were developed from local germplasm carrying PVY resistance genes (*pvr2*^{1} and *pvr2*^{2}) respectively using marker assisted selection (MAS). F_1 progeny were obtained by crossing a homozygous *pvr2*^{1} (resistant) 'Double Up' line with a heterozygous (*pvr2*$^{+}$/*pvr2*^{2}) susceptible 'Benno' cultivar. The F_1 and F_2 generations were assessed for the presence of PVY resistance alleles (*pvr2*$^{+}$/*pvr2*^{1}/*pvr2*^{2}) at the *pvr2-eIF4e* locus via the tetra primer amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) procedure. Negative selection was carried out using markers to detect the *pvr2*$^{+}$ (susceptible) allele. All F_1 progeny displaying the *pvr2*$^{+}$ allele were eliminated from the study. All 302 plants belonging to 29 F_2 families expressing homozygous recessive traits were tested via mechanical inoculation for their response to PVY infection and resistance to PVY was confirmed in all selected families based on symptomatology in greenhouse screens using double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA).

A total of 144 F_1 plants were genotyped as heterozygous (*pvr2*$^{+}$/*pvr2*^{1}) and 156 F_1 plants as (*pvr2*^{1}/*pvr2*^{2}). These results show that ARMS-PCR can be used to successfully screen pepper genotypes for alleles that confer PVY resistance thereby contributing to the improvement of pepper production.

4.1 Introduction

Potato virus Y (PVY) is an important pathogen that is naturally transmitted by several aphid species in a non-persistent manner (De Bokx and Huttinga, 1981; Shukla *et al.*, 1994). PVY infection substantially reduces the yield and quality of peppers (*Capsicum annuum* L) in susceptible varieties (Romero *et al*., 2001). Although the devastating effects of PVY have been more notable in warmer tropical and sub-tropical climates, bell-pepper industries all over the world have reported the typical mosaic symptoms associated with PVY infection (Green and Kim, 1991). In the KwaZulu-Natal (KZN) province of Republic of South Africa (RSA), PVY is a major constraint to pepper production (Budnick *et al*., 1996). Despite the fact that peppers are infected by approximately 45 viruses, tomato spotted wilt virus (TSWV), tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV) are the only other viruses that have been reported to infect pepper in RSA (Budnick *et al*., 1996). A recent field survey has confirmed that PVY is predominant among pepper farming communities in KZN and its surrounding areas (Ibaba and Gubba, 2011).

Current methods including cultural and vector control of PVY in KZN have partially controlled the spread of this disease. Therefore the development of resistant varieties (that possess desirable yield and quality traits) is the best alternative to manage PVY diseases on pepper. Moreover, the limited measure of control that may be achieved using prophylactic or cultural practices can become time consuming and expensive (Lecoq *et al*., 2004). Breeding techniques that incorporate PVY resistance genes into commercial pepper genotypes can now be optimized, as information relating to the type of gene action is better understood (Boiteux *et al*., 1996). However, the process of breeding virus resistant cultivars may be long and costly; therefore the source of resistance needs to be durable such that an effective degree of protection is maintained

throughout the commercial lifespan of the cultivar (Boiteux *et al*., 1996). Furthermore, there needs to be low cost markers available to track the resistance genes

Greenhouse screens revealed sources of PVY resistant pepper varieties in many parts of the world including South America, Europe and RSA (Pochard *et al*., 1983; Gebre-Selassie *et al*., 1985; Chapter 3 of this dissertation). The identification of naturally occurring resistance genes is important since they provide a highly efficient barrier to viral infection. Their recessive or dominant inheritance is strongly linked to underlying molecular mechanisms (Robaglia and Caranta, 2006). The development of resistant pepper varieties using conventional and molecular breeding techniques has been used for a considerable number of years (Kang *et al*., 2005) during which many resistant genes and quantitative trait loci (QTLs) have been identified. These include the *pvr1*, *pvr2* and *pvr5* resistance loci from *C. annuum* situated on chromosome P4 of pepper as well as the *pvr4* locus from *C. annuum* and *pvr7* locus from *C. chinense*, both occurring on chromosome P10 of pepper (Kyle and Palloix, 1997; Caranta *et al.*, 1997; Grube *et al.*, 2000; Kerlan, 2006).

In pepper, the $pvr2^1$ and $pvr2^2$ recessive alleles located at the *pvr2* resistance locus mediate pathotype specific resistance to PVY (Kyle and Palloix, 1997; Ruffel *et al*., 2002). These alleles have been effectively used to control PVY infection on pepper for more than half a century (Ben Khalifa *et al*., 2012). The $pvr2^1$ allele provides effective resistance against PVY pathotype 0 and the $pvr2^2$ allele is effective against pathotypes 0 and 1, however, both genes are overcome by the PVY pathotype 1-2 (Ruffel *et al*., 2002). A small number of described PVY isolates are able to overcome the resistance provided by the $pvr2^2$ allele and no resistance breakdown has been observed in the field which indicates the highly durable nature of this allele (Gebre-Selassie *et al*., 1985; Luis Arteaga *et al*., 1993). Although resistance breaking isolates have been reported in pepper crops that possess the $pvr2^1$ allele, they are less prevalent than avirulent types (Luis Arteaga and Gil Ortega, 1986; Ben Khalifa *et al*., 2012).

The *eIF4E* (translation initiation factor) has been recurrently detected in a variety of hosts as a naturally occurring recessively inherited resistance locus (Yeam *et al*., 2005). Genetic mutations occurring within the *eIF4E* initiation factor have been shown to confer

resistance to potyviruses in numerous plant species including pepper at the *pvr2* locus (Kang *et al*., 2005; Ruffel *et al*., 2002). Strong circumstantial evidence suggests that the relationship between *eIF4E* and viral protein VPg is fundamental for determining the outcome of potyvirus–host interactions (Yeam *et al*., 2005). Mutations in the *eIF4E* occurring at the *pvr2* locus results in gene products that are unable to interact with the viral protein Vpg (Kang *et al*., 2005). It is therefore possible that functional markers reported in literature are established on biologically meaningful single nucleotide polymorphisms (SNPs) that contribute to the phenotypic characteristic of interest (in this case susceptibility or resistance) (Yeam *et al*., 2005). Functional markers designed by Rubio *et al* (2008), uses tetra-primer ARMS-PCR (amplification refractory mutation system – polymerase chain reaction) to identify SNP signatures of four individual alleles at the *pvr2-eIF4E* locus (*pvr2*$^{+}$, *pvr2*^{1}, *pvr2*^{2} and *pvr2*^{3}) which regulates pepper resistance to PVY. Moreover, this technique is able to detect and differentiate both homozygous and heterozygous alleles in pepper genotypes.

The use of indirect selection via molecular genotyping methods facilitates the identification of desired alleles and haplotypes during the initial stages of the plant life cycle and early in the breeding line development which can ultimately lessen or eradicate cycles of phenotypic evaluation (Dubcovsky 2004; Frey *et al*., 2004). Marker-assisted selection (MAS) is especially significant when the trait is recessively inherited, polygenic or shows low heritability resulting from imprecise phenotype based selection (Yeam *et al*., 2005). Pepper crops in KZN are frequently infected by isolates of PVY belonging to the pathotype 0 group (Chapter 3 of this dissertation). Furthermore, the *pvr2*^{1} and *pvr2*^{2} recessive alleles provide effective resistance against PVY-pathotype 0 isolates. In this study, homozygous pepper lines were developed from locally available hybrids carrying PVY resistance genes (*pvr2*^{1} and *pvr2*^{2}) using marker-assisted selection (MAS).

4.2 Materials and Method

4.2.1 Virus isolate

The isolate of PVY used in this study was collected in the KZN province from infected pepper crops during field surveys. The isolate was positively identified using double antibody enzyme linked immunosorbent assay (DAS-ELISA) (ADGEN Phytodiagnostics, Europe). The ELISA positive isolate was propagated on *Nicotiana tabacum* cv. Xanthi leaves and stored frozen at -80°C for further use.

4.2.2 Crosses and pepper genotypes

Pepper lines Benno (susceptible) and Double Up (resistant) were used as parental lines. Thirty seeds from each parental line were sown in speedling trays containing finely milled pine bark (seedling mix) and maintained in a glass-house facility at the University of KwaZulu-Natal (UKZN). Three weeks post germination, these pepper seedlings were transplanted into 150 mm pots containing pine bark (potting mix). F_1 progeny were obtained by crossing a homozygous *pvr2*^{1} resistant line (Double Up) with a heterozygous susceptible line (Benno) carrying the *pvr2*$^{+}$ (susceptible) and *pvr2*^{2} (resistant) alleles. Benno was selected due to its early maturing medium blocky fruit set (Anonymous, 2008). Double Up has excellent yield potential, matures early and produces uniformly bright red blocky fruit (Anonymous, 2008). Many growers select for these traits based on demand for the intended market. The F_1 progeny were self-pollinated in an insect free glass-house facility under continuous sprinkle fertigation to obtain the F_2 seed.

4.2.3 DNA extraction

DNA was sampled from all F_1 and F_2 progeny using Indicating Whatman™ FTA™ Elute Cards (GE Healthcare Bio-Sciences, USA) according to the manufacturer's instructions. A section of fresh apical leaf tissue was pressed onto the membrane of the FTA card using a pestle for cell lysis and subsequent release of sap. To prevent carry over between samples, all materials used in the extraction procedure were sterilized with 70% ethanol after each sample. All samples were air dried at ambient temperature for 3 h before proceeding further. A 2 mm disc was punched out from the center of the dried sample area using a Harris uni-core micro punch and transferred into a 1.5 ml

microcentrifuge tube. To eradicate the possibility of cross-contamination between samples, a disc was punched out of a non-sampled area on the FTA card. After submerging the disc in 500-ul of sterile ultra-pure water, the sample was pulse vortexed 3 times for 5 s. The disc was carefully removed and transferred to a new 1.5 ml micro-centrifuge tube to which 30 ul of sterile Milli-Q water was added and incubated on a heating block at 95°C for 30 min. The sample disc was pulse vortexed for approximately 5 s and centrifuged at 10,000 x *g* for 1 min to elute the DNA. After carefully removing the disc with a sterile pipette tip, the eluted DNA was stored at -20°C.

4.2.4 Screening of progeny using marker assisted selection (MAS)

The F_1 and F_2 generations were assessed for the presence of PVY resistance alleles (*pvr2*$^{+}$/*pvr2*^{1}/*pvr2*^{2}) at the *pvr2-elF4e* locus using the tetra primer-amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) procedure described by Rubio *et al* (2008). Negative selection was carried out using the T200A, G325A and T236G tetra-primer sets to detect the *pvr2*$^{+}$ (susceptible) allele in 300 F_1 progeny. All F_1 progeny displaying the *pvr2*$^{+}$ (susceptible) allele were then eliminated from further study. Segregating F_2 progeny were genotyped using the G325A tetra-primer to differentiate homozygous *pvr2*^{1} and *pvr2*^{2} alleles. F_2 populations carrying the *pvr2*^{1} or *pvr2*^{2} recessive alleles were phenotyped in greenhouse screens. The annealing temperature and expected band size for each primer set is listed in Table 4.1. The tetra primer ARMS-PCR procedure was performed in 20 µl reactions which consisted of 10 ul KAPA 2G Fast DNA polymerase (Kapa Biosystems, Lasec, South Africa), 200 mM of each primer and 30 ng of genomic DNA. Conditions for ARMS-PCR was 95°C for 3 min; 35 cycles for 15 s at 95°C, 20 s at the annealing temperature specified for each set of primers listed in Table 4.1, 20 s at 72°C and a final elongation for 2 min at 72°C. Amplified DNA products were separated on a 1.5% agarose gel stained with SYBR safe (Invitrogen).

Table 4.1 Tetra-primer ARMS-PCR used to distinguish recessive alleles at the *pvr2-elF4E* resistance locus in pepper (Rubio *et al.,* 2008).

Primer	Sequence	Expected Size (bp)	Allele	Annealing Tm (°C)
T200A				
Forward Inner Primer	TCATGGACTTTCTGGTTTGATAATCCGGT	199	2^{+}	54
Reverse Inner Primer	CCAAGCAGCTTGTTTCGATTTCGTCT	258	2^{1},2^{2},2^{3}	54
Forward Outer Primer	TCCCGAAAGTAAAAAAAGCACACAGCAC	402		54
Reverse Outer Primer	TCGTGATTGTTCGATTCCCCTAATACCC	402		54
G325A				
Forward Inner Primer	CACCCAAGCAAGTTAGTTGTGGGAGAAA	198	2^{2}	48
Reverse Inner Primer	AATTTTATGCTTGAAACAATGTAATTC	288	2^{+},2^{1},2^{3}	48
Forward Outer Primer	GTACTTATGTGAATTTGGTGTCTGCCTT	431		48
Reverse Outer Primer	TACTAGAGTGACCAATCACTACGAGCTG	431		48
T236G				
Forward Inner Primer	GAAACAAGCTGCTTGGGGTAGCTCACT	176	2^{+},2^{3}	59
Reverse Inner Primer	CAACAGTGGAGAAAGTGTAGACGTTGCAAC	132	2^{1}.2^{2}	59
Forward Outer Primer	TGAGCAAAGAAATAGCAACAAAGCATCCA	251		59
Reverse Outer Primer	AAACTTCCACCTTGTTCGTGATTGTTCG	251		59

The forward and reverse outer primer for each tetra-primer set amplifies a common allele present in all pepper genotypes, whereas the forward and reverse inner primers are allele specific.

4.2.6 Mechanical inoculation

Mechanical inoculation was performed on 302 F_2 plants belonging to 29 families expressing homozygous recessive alleles (*pvr2*^{1}/*pvr2*^{1} or *pvr2*^{2}/*pvr2*^{2}) to test for their response to PVY infection. Artificial wounds were induced using carborundum powder as an abrasive. Virus inoculum was prepared from freshly harvested tobacco (*Nicotiana tabacum* cv. Xanthi) leaves previously infected with a pepper isolate (JVW-186; Chapter 2 of this dissertation) of PVY. Inoculum consisted of infected tobacco tissue homogenized in ice-cold 0.05 M sodium phosphate buffer (pH 7.4) (1 g of leaf tissue: 5ml buffer). Prior to inoculation (at the four leaf stage of development), leaves of F_2 progeny were lightly dusted with 400-mesh carborundum and manually rubbed with PVY inoculum. The inoculum was replaced every 10 min to ensure that the virus remained viable throughout the procedure. Inoculated leaves were rinsed with a jet of distilled water to remove excess inoculum. The inoculation procedure was repeated after seven days. Inoculated plants were maintained in an insect free glass house (26°C) and observed for virus symptom development for a period of eight weeks. The accumulation of PVY virions in host tissue was quantified using double antibody sandwich enzyme linked immunosorbent assay eight weeks post inoculation (DAS-ELISA) (ADGEN Phytodiagnostics, Europe).

4.3 Results

4.3.1 Screening the F_1 progeny

ARMS-PCR using three sets of tetra-primers listed in Table 4.1 was used to genotype 300 F_1 progeny at the *pvr2-eIF4E* locus in pepper crops that lead to PVY resistance (Appendix C). The band amplification patterns in Figure 4.1 - 4.3 show that the F_1 progeny were derived from the parental lines. The generation of a 256/431/402 bp common allele (present in all pepper genotypes depending on the respective tetra-primers) together with allele specific amplicons of the expected size was used to differentiate alleles in heterozygous F_1 genotypes. The amplification of a characteristic 176-bp and 132-bp fragment using the T236G tetra-primer shows the presence of *pvr2*$^{+}$; *pvr2*^{3} and *pvr2*^{1}; *pvr2*^{2} alleles respectively. The absence of the *pvr2*^{3} allele in parental

lines Benno and Double Up that were previously genotyped using tetra-primer ARMS-PCR reported in Chapter 3 of this dissertation indicated that all F_1 genotypes displaying the 176-bp amplicon were heterozygous *pvr2*$^{+}$/*pvr2*^{1} or *pvr2*$^{+}$/*pvr2*^{2}. F_1 progeny displaying the 132-bp fragment were heterozygous *pvr2*^{1}/*pvr2*^{2}.

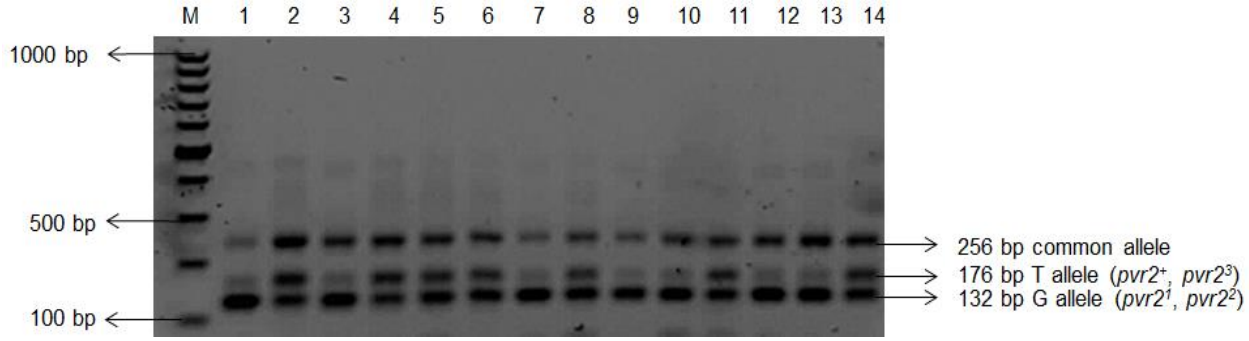

Figure 4.1 Agarose gel electrophoresis using tetra-primer (T236G) ARMS-PCR to differentiate heterozygous recessive alleles in F_1 progeny. Lane M: 100 bp ladder (Thermo Scientific); Lane 1: parental line Double Up (*pvr2*^{1}/*pvr2*^{1}); Lane 2: parental line Benno (*pvr2*$^{+}$/*pvr2*^{2}); Lanes 3-14: F_1 genotypes expressing heterozygosity at the *pvr2* locus.

Genotyping the F_1 progeny using the G325A tetra-primer (Fig. 4.2) generated a 288-bp fragment which indicates the presence of a *pvr2*$^{+}$, *pvr2*^{1} or *pvr2*^{3} allele. In addition, the amplification of a 198-bp fragment confirmed the presence of a *pvr2*^{2} allele. The absence of a *pvr2*^{3} allele in parental lines and subsequent F_1 individuals indicates that all F_1 progeny are heterozygous *pvr2*$^{+}$/*pvr2*^{1} or *pvr2*^{1}/*pvr2*^{2}.

Negative selection was carried out using the T200A marker to detect the susceptible *pvr2*$^{+}$ (199-bp amplicon) allele (Fig. 4.3). Although, the possibility of a *pvr2*$^{+}$ allele in heterozygous F_1 lines was also detected using the G325A (288bp amplicon; Fig. 4.2) and T236G (176-bp amplicon; Fig. 4.1) tetra-primer combinations, the T200A tetra-primer further confirmed the presence of the *pvr2*$^{+}$ allele in susceptible F_1 individuals.

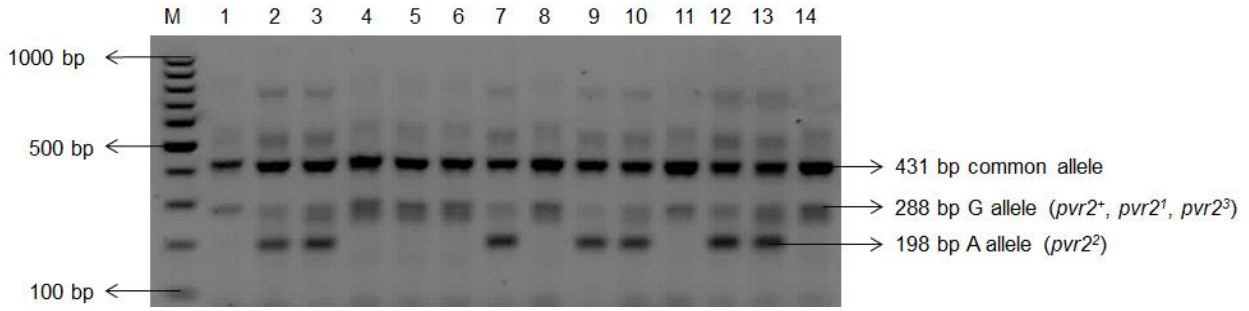

Figure 4.2 Agarose gel electrophoresis using tetra-primer (G325A) ARMS-PCR to confirm the presence of the *pvr2*^{2} recessive allele in heterozygous F_1 progeny. Lane M: 100 bp ladder (Thermo Scientific); Lane 1: parental line Double Up (*pvr2*^{1}/*pvr2*^{1}); Lane 2: parental line Benno (*pvr2*$^{+}$/*pvr2*^{2}); Lanes 3, 7, 9, 10, 12, 13: F_1 genotypes displaying the 198 bp-amplicon (*pvr2*^{2} allele). Lanes 4, 5, 6, 8, 11, 14: F_1 genotypes that are heterozygous *pvr2*$^{+}$ or *pvr2*^{1}.

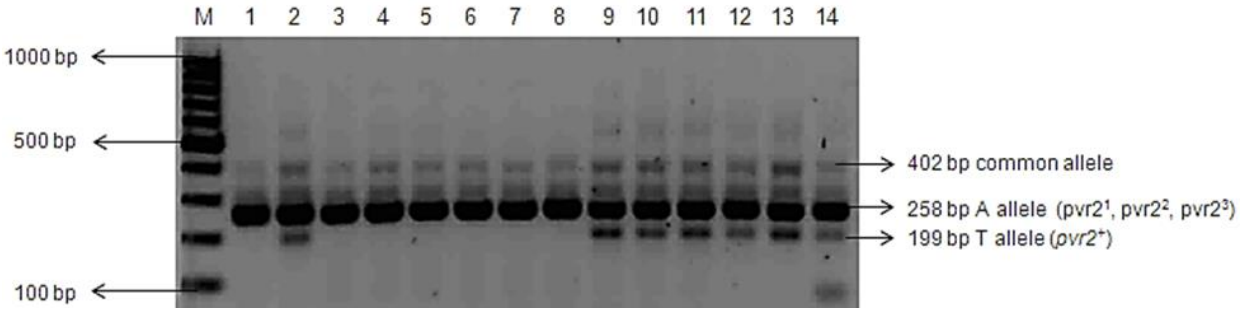

Figure 4.3 Agarose gel electrophoresis using tetra-primer (T200A) ARMS-PCR to identify the dominant *pvr2*$^{+}$ susceptible allele in F1 progeny. Lane M: 100-bp ladder (Thermo Scientific); Lane 1: parental line Double Up (*pvr2*^{1}/*pvr2*^{1}); Lane 2: parental line Benno (*pvr2*$^{+}$/*pvr2*^{2}); Lanes 3-8: F1 genotypes devoid of *pvr2*$^{+}$ allele; Lanes 9-14: F_1 genotypes displaying the *pvr2*$^{+}$ allele (199-bp amplicon)

Three hundred F_1 progeny were individually genotyped using the band amplification patterns generated from the tetra-primer ARMS-PCR procedure. The F_1 genotypes described in Appendix C, show that a total of 144 F_1 plants were identified as heterozygous (*pvr2*$^{+}$/*pvr2*^{1}) and 156 F_1 lines as (*pvr2*^{1}/*pvr2*^{2}). Subsequently, all

(*pvr2*^{1}/*pvr2*^{2}) heterozygous F_1 genotypes were self-pollinated to obtain the F_2 populations.

4.3.2 Screening the F_2 progeny

F_1 progeny carrying the *pvr2*^{1}/*pvr2*^{2} heterozygous recessive alleles were self-pollinated in glasshouse screens. All the F_2 individuals were genotyped as homozygous *pvr2*^{1}/*pvr2*^{1} or *pvr2*^{2}/*pvr2*^{2} using the G325A tetra primer (Fig. 4.4). The amplification band pattern displays the common allele (431-bp) and a subsequent 288-bp or 198-bp fragment that is homozygous *pvr2*^{1} or *pvr2*^{2} respectively.

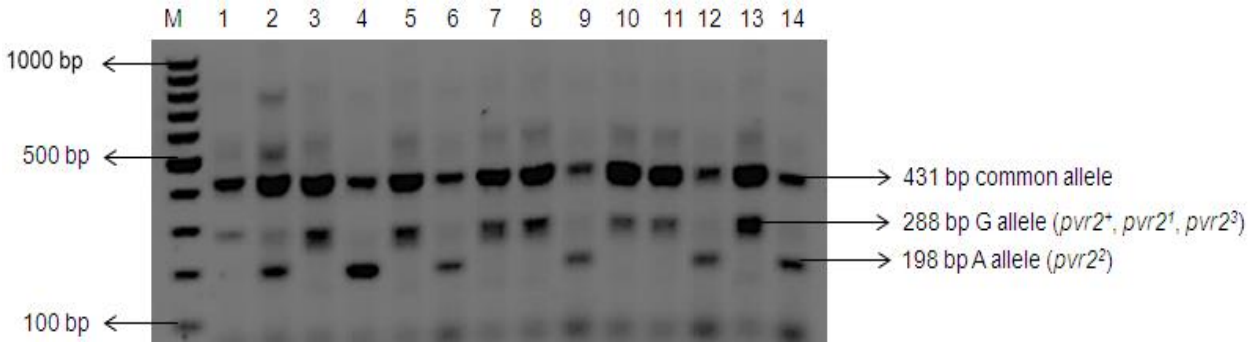

Figure 4.4 Genotyping the parental lines and F_2 progeny using tetra primer (G325A) ARMS-PCR. Lane M: 100 bp ladder (Thermo Scientific); Lane 1: parental line Double Up (*pvr2*^{1}/*pvr2*^{1}); Lane 2: parental line Benno (*pvr2*$^{+}$/*pvr2*^{2}); Lanes 3, 5, 7, 8, 10, 11 and 13: homozygous (*pvr2*^{1}/*pvr2*^{1}) F_2 progeny displaying the 288 bp amplicon; Lanes 4, 6, 9, 12 and 14: homozygous (*pvr2*^{2}/*pvr2*^{2}) F_2 progeny displaying the 198 bp amplicon.

Three hundred and two plants belonging to 29 F_2 families expressing recessive resistance alleles were tested via mechanical inoculation for their response to PVY infection. Resistance to PVY was determined in all selected families based on symptomatology in greenhouse screens. Eight weeks post inoculation, no symptoms were observed on any of the F_2 pepper crops; however Benno which was used as a positive control developed severe mottling, leaf distortion and stunting (Fig. 4.5). Phenotypic characteristics associated with F_2 resistant plants corresponded to the

homozygous genotypes in Figure 4.4. Absence of virus was confirmed in all F_2 individuals using DAS-ELISA (data not shown).

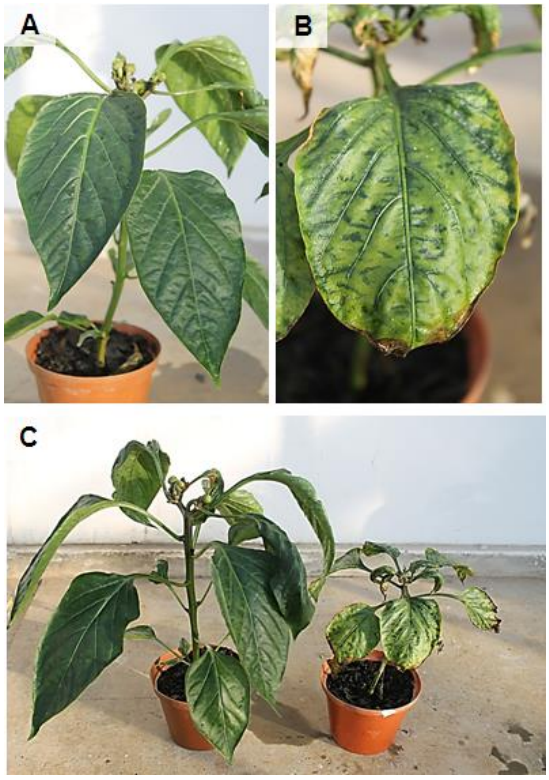

Figure 4.5 Phenotypic screening of F_2 progeny for resistance to PVY infection. Asymptomatic leaves of F_2 pepper plant after mechanical inoculation (A); Single leaf from the susceptible parent (Benno) showing severe vein banding and chlorosis (B); Comparison of a resistant F_2 plant which is symptomless with a susceptible parent (Benno) expressing severe mottling, stunting and leaf distortion (C).

4.4 Discussion

Recessive alleles (*pvr2*$^{+}$, *pvr2*^{1}, *pvr2*^{2} and *pvr2*^{3}) located at the *pvr2* locus are found to control resistance or susceptibility to isolates of PVY in pepper genotypes (Ben Khalifa *et al*., 2009). Therefore it is necessary to identify the infecting PVY pathotype/s prior to selecting the appropriate parental genotypes in breeding programs. Three PVY

pathotypes, designated (0), (0-1) and (1-2) were defined according to their ability to overcome the recessive resistance alleles located at the *pvr2* locus in pepper (Gebre-Selassie *et* al., 1985). The *pvr2*^{1} allele provides effective resistance against PVY pathotype 0 and the *pvr2*^{2} allele is effective against pathotypes 0 and 1; however both genes are overcome by the PVY pathotype 1-2 (Ruffel *et al*., 2002). Caranta *et al* (1997) shows that the *pvr2*^{3} allele confers resistance to pathotypes (0) and (1-2). However, the *pvr2*^{3} recessive allele was not identified in any of the pepper lines used in this study (Chapter three of this dissertation). Ruffel *et al* (2004) showed that all these alleles correspond to the eukaryotic translation initiation factor *eIF4E* and differ by a few amino acid substitutions.

The *eIF4E* (translation initiation factor) has been recurrently detected in a variety of hosts as a naturally occurring recessively inherited resistance locus (Yeam *et al*., 2005). Genetic mutations occurring within the *eIF4E* initiation factor have been shown to confer resistance to potyviruses in numerous plant species including pepper at the *pvr2* locus (Kang *et al*., 2005; Ruffel *et al*., 2002). Strong circumstantial evidence suggests that the relationship between *eIF4E* and viral protein VPg is fundamental for determining the outcome of potyvirus–host interactions (Yeam *et al*., 2005). Mutations in the *eIF4E* occurring at the *pvr2* locus results in gene products that are unable to interact with the viral protein Vpg (Kang *et al*., 2005). It is therefore possible that functional markers reported in literature are established on biologically meaningful SNPs that contribute to the phenotypic characteristic of interest (in this case susceptibility or resistance) (Yeam *et al*., 2005).

Functional markers designed by Rubio *et al* (2008) were used in this study to distinguish recessive alleles of all F_1 and F_2 progeny at the *pvr2-eIF4E* resistance locus in pepper using the tetra primer ARMS-PCR procedure. Allele specific codominant markers are useful to assay recessive alleles in heterozygous progenies which cannot be achieved through phenotypic evaluations (Rubio *et al*., 2008). The isolate of PVY (JVW-186) infecting pepper crops in KZN was classified as pathoype 0 (Chapter 3 of this dissertation) based on its ability to overcome recessive resistance alleles (*pvr2*$^{+}$, *pvr2*^{1} and *pvr2*^{2}) at the *pvr2* locus, previously described by Gebre-Selassie *et al* (1985).

Therefore successful infection of JVW-186 can only occur in pepper crops that have a *pvr2*$^{+}$ allele. Interestingly, the presence of a *pvr2*^{2} allele in conjunction with a *pvr2*$^{+}$ allele in the parental line Benno did not provide resistance to the PVY pathotype (JVW-186) after mechanical inoculation tests.

According to Ben Khalifa *et al* (2009), the *pvr2*$^{+}$ allele is found to be dominant for susceptibility in the presence of resistance alleles in heterozygous pepper crops. Subsequently, all F_1 progeny displaying the *pvr2*$^{+}$ allele were eliminated from the study. Furthermore, pathotype 0 isolates of PVY can only infect pepper crops with a *pvr2*$^{+}$ allele (Gebre-Selassie *et al*., 1985), which corresponds with the predominant pathotype infecting pepper in KZN (Chapter 3 of this thesis). Therefore, only heterozygous F_1 hybrid peppers carrying the *pvr2*^{1}/*pvr2*^{2} alleles were retained and self-pollinated to generate segregating F_2 progeny. Genotyping the F_2 population using the G325A tetra-primer revealed homozygous pepper crops carrying the *pvr2*^{1}/*pvr2*^{1} or p*vr2*^{2}/*pvr2*^{2} recessive resistance alleles. According to Rubio *et al* (2008), only one set of primers are required to genotype bi-parental populations in which the parent genotypes are known.

Three hundred and four F_2 plants were phenotyped using the infecting PVY isolate JVW-186 to infer their genotype from their phenotype. All resistant F_2 pepper plants corresponded with the recessive homozygous genotypes (*pvr2*^{1} or *pvr2*^{2}). The use of DAS-ELISA failed to detect the presence of PVY virus particles in asymptomatic F_2 progeny (data not shown). The inability to detect the presence of PVY virions in previously inoculated host tissue confirmed that the *pvr2*^{1} and *pvr2*^{2} recessive alleles in F_2 pepper crops individually mediate resistance to the pathotype of PVY infecting peppers in KZN.

According to Greenleaf (1986), despite the extensive cultivation of *pvr2*^{1} and *pvr2*^{2} carrying pepper cultivars throughout the world, virulence of PVY isolates toward the *pvr2*^{1} was not predominant and very rarely observed toward the *pvr2*^{2} (Boiteux *et al*., 1996). This indicates that the *pvr2*^{2} allele provides a higher level of durability against a wider range of PVY isolates. Although the *pvr2*^{3} allele was shown to confer resistance to various pathotypes of PVY (Caranta *et al*., 1997) it has not been

introgressed into pepper hybrid cultivars (Ayme *et al*., 2007). Furthermore, the frequency of virulent PVY isolates toward the *pvr2*^{3} recessive allele suggests that the durability of this allele is questionable (Ayme *et al*., 2006). Taken together, it can be inferred that pepper varieties carrying the *pvr2*^{2} allele are less likely to experience resistance breakdown in the field.

Although the *pvr2*^{1} and *pvr2*^{2} homozygous pepper varieties developed in this study are equally effective against infection from the isolate of PVY (JVW-186) in KZN, resistance breakdown of the *pvr2*^{1} has been reported in pepper crops from other parts of the world (Luis Arteaga and Gil Ortega, 1986; Ben Khalifa *et al*., 2012). Consequently, the *pvr2*^{2} homozygous pepper variety provides resistance to a wider range of PVY isolates. The durable nature of this resistance is attributed to the absence of resistance breakdown from field reports (Gebre-Selassie *et al*., 1985; Luis Arteaga *et al*., 1993). The small number of naturally occurring PVY isolates able to overcome the resistance mediated by the *pvr2*^{2} allele, in-addition to the unusually low frequency of naturally occurring *pvr2*^{1} resistance-breaking isolates, suggests that the homozygous pepper lines developed in this study will provide a substantial level of resistance to PVY infection.

These results show that ARMS-PCR can be used to successfully screen pepper genotypes for alleles that confer PVY resistance thereby contributing to the improvement of pepper production in KZN and abroad. Furthermore, these markers eliminate the need for expensive, labour-intensive marker studies and progeny testing usually associated with recessively inherited traits in conventional breeding (Rubio *et al*., 2008). In order to effectively manage the pepper cultivars for durable PVY resistance, heterozygous pepper plants carrying *pvr2*^{1}/*pvr2*^{2} were not developed. The risk of accumulating resistance is associated with selection pressure exerted by one allele that can accelerate the acquisition of virulence toward the other (Ayme *et al*., 2007). The homozygous pepper genotypes obtained in this study provide a stable form of resistance to the KZN isolate of PVY (JVW-186; Chapter 2 of this dissertation). The genetic resources can be exploited by breeders and introgressed into commercially available pepper varieties.

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Chapter 5

Dissertation Overview

Peppers are grown and consumed throughout most parts of the world and their annual production for use as a spice or vegetable has significantly increased over the years. The nutritional value of the crop as a source of highly concentrated antioxidants (vitamin C, E and provitamin A) has gained popularity among many populations of the world (Bosland *et al*., 2012). In addition, compounds such as capsaicin derived from pepper extracts are used in pharmacology and modern medical applications to alleviate pain and inflammation (Bosland *et al*., 2012). Bell peppers are consumed raw or cooked; and adds flavor to a wide range of culinary dishes such as salads, soups, and fries. The importance of this crop in the 21st century is being recognized for use in a wide range of applications that are supplementary to the food, medical and health industries which has spurred voracious interest among the general public (Bosland *et al*., 2012).

Economic losses incurred by global pepper industries as a result of infection by pathogenic agents such as PVY warrants the development of efficient strategies to protect these crops. Potato virus Y (PVY) is a serious disease of pepper in the province of KwaZulu-Natal (KZN) in Republic of South Africa (RSA) and other parts of the world (Budnik *et al*., 1996; Ibaba and Gubba, 2011). The highly variable nature of this pathogen requires control strategies that are durable against the naturally existing pathotypes of PVY in a specific area. In a country like RSA, poverty is rife and subsistence farming is a common practice among rural communities. Since pepper is a high value crop, it affords rural communities the opportunity to earn a source of income. PVY outbreaks across the province have the greatest economic impact on resource poor farming communities. Many of these people suffer due to the lack knowledge and resources for plant disease management. Many practiced protocols to mitigate the incidence of PVY have not provided a sufficient measure of control. It is therefore important to have an in-depth study of PVY infecting pepper in KZN so as to effectively manage the disease it causes on the crop.

5.1 Major Findings

The findings presented in this study are based on the characterization and whole genome sequencing of a PVY isolate infecting pepper in KZN, screening of different pepper lines for resistance to PVY and the use of molecular breeding as a strategy to sustainably manage PVY on pepper.

In Chapter 2, the first complete genome sequence of a pepper-infecting isolate of PVY was reported from KZN RSA. The complete sequence alignment of the PVY JVW-186 isolate compared with 10 parental strains comprised 9700-bp with two recombinant breakpoints at position 343 and 9608-bp. JVW-186 is a recombinant PVY pathotype which causes severe mosaic and mottling symptoms in susceptible tomato and tobacco varieties. Phylogenetic analysis revealed that the first recombination event contained a fragment that clustered with PVY isolates in the C clade. The second recombination junction occurred in the region of the coat protein and clustered with PVY^{O} parental isolates which explains the positive reaction with PVY^{O}-specific antibodies. The large non-recombinant 7942 bp segment did not cluster with PVY isolates from any of the clades. The unique 7942 bp segment suggests the possibility of a novel strain of PVY that has evolved due to selection pressure.

According to sequence data analysis using RDP4 in combination with phylogenetic analysis regions of the genome sequence of this isolate may belong to a completely different strain group of PVY. The high level of nucleotide sequence similarity of individual non-recombinant and recombinant segments of the sequence alignment shared among the various PVY groups, together with the identification of typical potyvirus-like virions using transmission electron microscopy and a 30 kDa coat protein from the SDS-PAGE protein assay confirmed that JVW-186 is indeed an isolate of PVY and the possibility of a novel recombinant strain. In addition, two ORF's identified at positions 186 and 2915 of the sequence alignment is the site of polyprotein synthesis and the frameshift translation protein P3N-PIPO respectively. The identification of these ORF's in conjunction with ten functional proteins at various coding regions across the JVY-186 sequence alignment is typically associated with potyviruses.

Results reported in Chapter 3 on the evaluation of different commercially available pepper lines revealed two sources of resistance. All susceptible pepper lines carrying the *pvr2*$^{+}$ allele corresponded with the development of symptoms which confirmed that the *pvr2*$^{+}$ allele is dominant for susceptibility to PVY. The resistant lines Double Up and Cecelia were genotyped as homozygous lines carrying the respective *pvr2*^{1} and *pvr2*^{2} recessive alleles and remained symptomless throughout the evaluation. The strain of PVY was unable to overcome the resistance genes *pvr2*^{1} and *pvr2*^{2} identified in Double Up and Cecelia respectively which indicates that the strain of PVY infecting pepper in KZN belongs to PVY- pathotype 0. The homozygous sources of resistance identified in pepper lines Cecelia and Double-Up during this study can be used in breeding programs intended to control PVY infecting pepper in KZN.

Chapter 4 demonstrates that tetra-primer ARMS-PCR can be used to successfully screen pepper genotypes for alleles that confer PVY resistance thereby contributing to the improvement of pepper production in KZN and other parts of the world. Failure to detect the presence of PVY virions in previously inoculated host tissue confirmed that the *pvr2*^{1} and *pvr2*^{2} recessive alleles in F_2 pepper crops individually mediate resistance to the pathotype of PVY infecting peppers in KZN. The small number of naturally existing PVY isolates able to overcome the resistance provided by the *pvr2*^{2} allele coupled with an unusually low frequency of naturally occurring *pvr2*^{1} resistance-breaking isolates, provide a stable resistance to PVY infection in KZN. In-order to effectively manage the pepper cultivars for durable PVY resistance, heterozygous pepper plants carrying *pvr2*^{1}/*pvr2*^{2} were not developed. The risk of accumulating resistances is associated with selection pressure exerted by one allele that can accelerate the acquisition of virulence toward the other.

5.2 Way Forward

The pathogenicity and host range of this PVY isolate should be tested on various potato cultivars and other important solanaceous crops such as eggplant/brinjal (*Solanum melongena*). This information may add substance to the evolutionary history of the isolate in terms of recombination. The fact that a large non recombinant region of

the of the sequence alignment of JVW-186 did not cluster within the clades of the three known PVY groups merits further analysis of the corresponding amino acid residues to confirm the likelihood of an ancestral PVY lineage.

A wider range of pepper genotypes can be assayed at the *pvr2-eIF4E* locus to identify alleles that confer resistance to all pathotypes of PVY. Charron *et al* (2008) described a total of ten recessive allelic varients at the *pvr2* locus which includes the $pvr2^{+}$ (susceptible allele), $pvr2^{1}$, $pvr2^{2}$, $pvr2^{3}$, $pvr2^{4}$, $pvr2^{5}$, $pvr2^{6}$, $pvr2^{7}$, $pvr2^{8}$, $pvr2^{9}$ that mediate pathotype specific resistance against PVY. Their study showed that the $pvr2^{5}$, $pvr2^{6}$, $pvr2^{7}$, $pvr2^{8}$, $pvr2^{9}$ provide a stable form of resistance to all pathotypes of PVY infecting pepper. Analysis of the interaction between the *eIF4E* locus resistance proteins and VPg ligand will provide insight into their co-evolution in terms of selection pressure. A profile of the non-synonymous amino acid substitutions in the *eIF4E* and VPg will contribute to the development of durable resistance against PVY.

A survey/field trial to determine actual percentage yield losses of pepper infected at different stages of growth with PVY in KZN is also required.

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Appendix A

SDS-PAGE stock solutions

Solution A: Monomer Solution [30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide]

Acrylamide (14.0g)

Bis-acrylamide (0.4g)

Dissolved and made up to 50ml with distilled water

Stored in an amber coloured bottle at 4^{o}C.

Filtered through Whatman No. 1 filter paper before use

Solution B: 4x Running Gel Buffer [1.5M Tris-HCl, pH 8.8]

Tris (9.070g)

Dissolved in 40ml distilled water

Adjust to pH 8.8 with HCl

Made up to 50ml

Filtered through Whatman No. 1 filter paper before use

Solution C: 4x Stacking Gel Buffer [500mM Tris-HCl, pH 6.8]

Tris (3g)

Dissolved in 20ml distilled water

Adjust to pH 6.8

Made up to 25ml

Filtered through Whatman No. 1 filter paper before use

Solution D: 10% (m/v) SDS

SDS (2.5g)

Dissolved in 25ml distilled water with gentle heating

Solution E: Initiator [10% (m/v) ammonium persulfate]

Ammonium persulfate (0.1g)

Made up to 1 ml just before use

Solution F: Tank Buffer [250mM Tris-HCl, 192mM glycine, 0.1% (m/v) SDS, pH 8.3]

Tris (6g)

Glycine (14.4g)

Made up to 1 liter with distilled water

2.5ml of solution D was added to 250ml prior to use

Solution G: Reducing Treatment Buffer [125mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]

Solution C (0.25ml)

Solution D (0.4ml)

Glycerol (0.2ml)

2-mercaptoethanol (0.1ml)

Made up to 1ml in distilled water

Appendix B

Nucleotide composition of JVW-186 sequence alignment

1 AAATTAAAAC AACTCAATAC AACATAAGAG AAACAACGCA AAAACACTC CAAACGCTTA

61 TTTGCAATCA AGCAACTTAC TAAGTTTCAG CTTTAATCTT TTTCTTGCAA TTTTCTGTAA

121 CGATATTAAG AATTACTCTA GTTTAACAAG CCATTTCATC TCTTTTAACT ATTAGCATAT

181 TCTCTATGGC AACTTACATG TCAACAGTTT GTTTTGGTTC AATTGAATGT AAGCTACCAT

241 ACTCACCCGC CTCTTGCGGA TATGTCACTA AGACAAGAGA GGTGCCGACT CCTGTCGACC

301 CTTTTGCGAA CCTGGAGGCA AAGCTTCAAA CACGACTAGC CAGGCAAAAA TATGCAACTG

361 TTCGTGTATT GAAGAACGGT ACTCGTGTAT ACCGATACAA GACTGATGCG CAGATAGTGC

421 GTATTCAGAA GAAATTGGAA AGGAAAGAGA GAGATGAGTA TCACTTTCAG ATGGCTGCTC

481 CAAGTATTGT GTCAAAGATT ACCATTGCTG GTGGAGATCC CCCATCAAAG TCTGAACCAC

541 AAACGCCGAA AGGAGTTATT CACACAACTC CAAGGGTGCG TAAAGTTAAG ACACACTCCA

601 TACCAAAGCT GACGGAAAGT CAAATGAATC ACCTCATCAA GCAGGTGAAG CGAATAATGT

661 CGGCTAAGAA AGGGTCTGTC CACCTAATTA ATAAGAAAAG TACGCACGTT CAATATAAGG

721 AAATACTTGG AACAACTCGC GCAACTGTTC GTACTGCACA TATGGTGGGC TTGCGACGAA

781 GAGTGGACTT CCGATGTGAT ATGTGGACGA TCAAGTGTTT GAAATGTCTC GCTCGAACAG

841 ATAAATGGTC GAATCAAGTT CGCACCTTCA ACATACGAAA GGGCGATAGT GGTGTCATCT

901 TGAATGCTGA CAGCCTCAAA GGTCACTTTG GGAGAAGTTC GGAAGGTTTG TTTATAGTGC

961 GTGGATCACA TGAAGGTAAA TTATATGACG CACGTTCCAA GGTTACTCAA GGTGTATTGA

1021 ACTCGATGAT TCAGTTCTCG AATGCTGAGA ATTTTTGGGA TGGTCTGGAC GGCAACTGGG

1081 CACAGATGAG ATACCCTTCA GATCACACAT GTGTAGCTGG TATACCAGTC GAGGACTGCG

1141 GTAGAATCGC TGCATTGATG ACACACAGTG TTTTGCCGTG CTACAAGATG ACCTGCCCCA

1201 CCTGCGCTCA ACAATATGCT AACTTGCCAA CCAGCGACTT ACTCAAGCTG TTGCATAAAC

1261 ATGCAAGGGA TGGCTTGAGT CGATTGGAAT CGGATAAAGA TCGATTCATG CATGTGAATA

1321 AGTTCTTGGC AACGTTGGAG CATTTAACTG AACCAGTTGA TCTAAGTCTT GAGCTTTTCA

1381 ATGAGATATT TAAATCCATA GGAGAGAAGC AGCAGGCACC ATTCAAGAAT TTAAATGTTT

1441 TAAATAATTT CTTTTTGAAA GGAAAAGAAA ACACAGCTCA TGAATGGCAG GCAGCTCAAT

1501 TGAGTCTGCT TGAATTAGCG AGATTCCAGA AGAACAGAAC TGATAACATC AAGAAAGGCG

1561 ATATTTCTTT CTTCAGAAAT AAATTATCTG CCAAAGCGAA TTGGAATCTA TATTTGTCGT

1621 GCGACAATCA GCTGGATAAA AATGCGAACT TCCTGTGGGG ACAAAGGGAA TATCATGCTA

1681 AGCGGTTTTT CTCAAACTTC TTTGAGGAAA TTGATCCAAC AAAGGGATAC TCAGCATATG

1741 AAATCCGCAA GCATCCGAAC GGAACGAGAA AGCTCTCAAT TGGTAACTTG ATTGTCCCAC

1801 TTGATTTGGC TGAATTTAGG CAGAAGATGA AAGGTGATTA TAGGAAACAA CCAGGTGTCA

1861 GCAAGAAGTG CACGAGCTCA AAGGATGGTA ATTATGTGTA TCCCTGCTGT TGCACAACAC

1921 TTGATGATGG TACAGCCATC GAGTCGACAT TCTATCCGCC AACCAAAAAG CACCTTGTGA

1981 TAGGCAATAG TGGTGATCAA AAGTTTGTTG ATTTACCAAA AGGAAATTCG GATATGCTGT

2041 ACATTGCCAA GCAGGGTTAC TGTTATATCA ACGTGTTTCT TGCGATGTTG ATTAACATTA

2101 GTGAGGAGGA TGCAAAGGAC TTCACAAAGA GAGTTCGCGA CATGTGTGTG CCAAAGCTTG

2161 GAACCTGGCC AACGATGATG GATCTGGCAA CTACTTGCGC TCAATTGAAA ATATTCTATC

2221 CAGACGTGCA TGACGCAGAA TTGCCCAGAA TATTGGTTGA TCATGACACT CAAACGTGTC

2281 ATGTAGTCGA CTCATTTGGT TCGCAAACAA CCGGATACCA TATTCTAAAA GCATCCAGTG

2341 TGTCTCAACT AATTCTGTTT GCAAATGATG AGCTAGAATC TGATATCAAG CATTACAGAG

2401 TTGGTGGTGT TCCGAATGCA TGTCCTGAAC TTGGATCTAC AATATCACCT TTTAGAGAAG

2461 GAGGATGCAT AATGTCTGAG TCGGCAGCAC TTAAACTGCT TCTAAAAGGG ATTTTCAGAC

2521 CCAAGGTGAT GAGAAAGTTG CTGTTGGACG AGCCTTACTT GTTGATATTG TCAATATTAT

2581 CCCCTGGCAT ACTGATGGCT ATGTATAATA ATGGGATTTT TGAGCTTGCG GTTAGGCCGT

2641 GGATCAATGA GAAACAATCA ATAGCCTTGA TAGCATCACT ACTATCAGCC TTGGCTCTAC

2701 GAGTGTCAGC AGCAGAGACA CTCGTCGCAC AGAGGATTAT AATCGATGCT GCAGCCACAG

2761 ATCTTCTTGA TGCAACATGT GACGGGTTCA ATCTATATCT AACGTACCCC ACTGCACTAA

2821 TGGTGTTGCA GGTTGTTAAG AATAGAAATG ATTGTGATGA TACCCTATTC AAGGCGGGTT

2881 TTCCAAGTTA CAACATGAGC GTTGTGCAAA TTATGGAAAA AAATTATCTA AATCTCTTGG

2941 ACGATGCTTG GAAAGATTTA ACTTGGCGGG AAAAATTGTC CGCAACATGG CATTCATACA

3001 GAGCAAAACG CTATATCACT CGGTATACCA AACCCACAGG AAAAGCAGAT TTGAAAGGGT

3061 TATACAACAT ATCACCACAA GCATTCTTGG GTCGAGGCGT TCAGAAGGCC AAAGGCACTG

3121 CTTCAGGCTT GAGCGAGCAG TTTAATAATT ATTTGAATAC TAAGTGTGTA AATATTTCAT

3181 CCTATTTCAT TCGTAGAATT TTTAGGCGCT TGCCGACTTT TGTTAACTTT GTAAATTCAT

3241 TATTAGTTAT TAGTATGTTA ACTAGTGTAG TAGCAATGTG TCAGTCGATA ATCTTGGATC

3301 AGAGGAGGTA TAAGAAGCAG ATTGAGTTGA TGCAGATTGA GAAGAATGAG ATTGTCTGTA

3361 TGGAACTATA TGCAAGTTTG CAGCGCAAGC TTGAGCGTGA TTTCACATGG GATGAGTATA

3421 TAGAATACTT GAAGTCAGTG AACCCTCAGA TAGTTCAATT TGCACAAGCA CAAATGGAGG

3481 AATACGATGT GCGACATCAG CGTTCCACAC CAGGTGTGAA AAATTTGGAA CAAGTGGTAG

3541 CATTTATGGC TTTGGTGATC ATGGTGTTTG ATGCCGAAAG GAGTGATTGT GTTTTTAAAA

3601 CTCTCAACAA ATTCAAGGGT GTTCTTTCCT CACTCGATCA CGAAGTAAGA CATCAGTCCT

3661 TAGATGATGT GATCAAGAAC TTTGATGAGA GAAATGAGGT TATAGATTTC GAGTTAAGTG

3721 AGGACACAAT TCGAACGTCA TCAGTGCTCG ATGTTAAGTT TAGTGATTGG TGGGACCGGC

3781 AAATTCAGAT GGGACACACA CTTCCACATT ACAGAACTGA GGGGCATTTC ATGGAATTTA

3841 CAAGAGCAAC AGCTGTCCAA GTGGCTAATG ATATTGCCCA TAGTGAACAT CTAGACTTTC

3901 TGGTGAGGGG AGCAGTAGGA TCTGGTAAGT CTACTGGGTT ACCTGTTCAT CTTAGTGTGG

3961 CGGGATCTGT GCTTTTGATT GAACCAACGC GACCATTAGC AGAGAATGTT TTCAAACAGC

4021 TATCTAGTGA ACCATTCTTT AAGAAACCAA CGCTTCGCAT GCGCGGAAAT AGTATTTTTG

4081 GCTCATCCCC AATTTCTGTT ATGACTAGCG GATTCGCATT GCATTATTTT GCTAATAATC

4141 GCTCCCAGCT AACTCAGTTT AACTTCATAA TATTCGATGA GTGCCATGTT CTAGACCCTT

4201 CCGCAATGGC TTTTCGTAGC TTATTGAGTG TTTATCACCA AGCATGCAAG GTGCTTAAAG

4261 TGTCAGCCAC TCCAGTGGGA AGGGAAGTCG AATTCACGAC ACAGCAGCCA GTCAAGTTAA

4321 TAGTGGAGGA TTCACTGTCT TTTCAATCCT TTGTTGATGC ACAAGGTTCT AAAACTAATG

4381 CTGATGTTGT TCAGTATGGT TCAAACATAC TTGTGTACGT GTCGAGCTAT AATGAAGTTG

4441 ATGCCTTGGC GAAACTCCTA ACAGACAAAA ATATGATGGT TACAAAGGTT GATGGCAGAA

4501 CAATGAAGCA TGGTTGTCTA GAAATAGTCA CAAAAGGAAC CAGTGCAAAA CCACACTTTG

4561 TTGTAGCAAC CAACATAATC GAGAATGGAG TAACTCTGGA CATAGATGTG GTTGTGGATT

4621 TTGGGCTCAA GGTGTCACCA TTTCTAGACA TCGATAATAG GAGCATTGCT TACAACAAGG

4681 TTAGTATTAG CTACGGTGAA AGGATTCAGA GGTTGGGTCG TGTTGGACGC TTCAAGAAAG

4741 GAGTGGCATT ACGCATTGGA CACACTGAGA AGGGAATCAT TGAAATTCCA AGTATGATTG

4801 CCAGTGAAGC TGCCCTTGCT TGCTTTGCAT ACAACTTGCC AGTAATGACA GGAGGAGTTT

4861 CAACTAGTCT AATCGGTAAT TGTACTGTGC GTCAGGTTAA AACAATGCAA CAATTTGAAT

4921 TGAGTCCCTT CTTTACTCAG AATTTTGTTG CTCATGATGG ATCAATGCAC CCTGTCATAC

4981 ATGACATTCT AAAGAAGTAT AAACTTCGAG ATTGCATGAC ACCTCTGTGT GACCAGTCTG

5041 TACCATATAG AGCCTCAAGT ACTTGGCTGT CAGTGAGTGA ATATGAGAAA CTTGGGGTGG

5101 TCCTAGATAT TCCAAGTCAC TTTAAAGTTG CATTTCATGT TAAGGATATT CCTCCCAAAC

5161 TCCACGAAAT GCTATGGGAA ACGGTTGTTA AGTACAAAGA TGTCTGTTTG TTTCCAAGCA

5221 TTCGCGCATC TTCCATCAGC AAAATTGCAT ACACATTGCG CACAGATCTT TTTGCTATTC

5281 CAAGAACTTT AATATTAGTG GAGAGATTAC TCGAAGAAGA GCGAGTGAAG CAGAGCCAAT

5341 TCAGAAGTCT TATTGATGAA GGATGTTCAA GCATGTTCTC AATTGTTAAC TTGACTAACA

5401 CTCTTAGAGC TAGATATGCA AAAGACTACA CTGCAGAGAA CATACAAAAG CTTGAGAAAG

5461 TGAGAAGTCA GTTAAAAGAA TTCTCAAACT TAGATGGCTC TGCATGTGAG GAGAATTTAC

5521 TGAAGAGGTA CGAGTCTTTG CAGTTTGTCC ATCATCAGTC TACGACATCA CTCGCAAAGG

5581 ATCTTAAGCT GAAGGGAGTT TGGAAGAAGT CATTAGTGGC CAAAGACTTG CTCATAGCAG

5641 GAGCCGTTGC AGTTGGTGGA GTAGGGCTCA TATATAGCTG GTTCACACAA TCGGTTGAAA

5701 CCGTGTCCCA CCAAGGGAAA AATAAATCCA AAAGAATTCA AGCTTTGAAG TTTCGCCATG

5761 CTCGCGACAA AAGGGCTGGT TTTGAAATTG ACAATAATGA TGATACAATT GAGGAATTCT

5821 TTGGATCTGC ATACAGAAAG AAGGGAAAAG GTAAAGGCAC CACAGTTGGC ATGGGCAAGT

5881 CAAGTAGGAG GTTTATCAAC ATGTATGGGT TTGATCCAAC AGAGTATTCG TTCATTCAAT

5941 TCGTTGACCC ACTCACTGGA GCACAAATAG AAGAGAATGT TTATGCTGAC ATTGGAAACA

6001 TTCAAGAAAG ACTTAGTGAA GTGCGAAAGA AAATGGTCGA GGACGATGAA CTCGAAGTGC

6061 AAGCCTTGGG AAACAATACG ACTATACATG CATACTTCAG AAAGGACTGG TCTGACAAAG

6121 CTTTGAAGAT TGACCTAATG CCACACAATC CGCTTAAGAT TTGTGATAAA ACAAATGGTA

6181 TTGCCAAATT TCCTGAGAGA GAGTTTGAAT TGAGGCAGAC TGGGCCAGCT GTAGAAGTCG

6241 ATGTGAAGGA TATACCGAAG CAGGATGTAG AGCATGAAGC TAAATCGCTC ATGAGGGGTT

6301 TGAGAGATTT TAACCCAATT GCCCAAACGG TTTGTAGGTT GAAAGTATCT GCTGAACATG

6361 GAACAACAGA AGTGTATGGT GTCGGGTTTG GGGCCTACAT AATAGCAAAT CACCATTTGT

6421 TCAAGAGCTA CAATGGTTCA ATGGAGGTGC GATCTATGCA TGGCACGTTT AGGGTGAAAA

6481 ACCTACACAG TTTGAATGTT TTGCCGATTA AGGGTAGGGA CATCATCCTC ATCAAAATGC

6541 CAAAAGATTT TCCTGTTTTC CCACAGAAGT TACGTTTCCG AGCTCCAACA CAGAATGAAA

6601 GGATTTGTTT AGTTGGAACA AATTTTCAAG AGAAGTACGC ATCTTCAATC GTCACAGAAA

6661 CTAGCACTAC TTACAATGTA CCAGGTAGCA CTTTTTGGAA GCATTGGATT GAAACAGATG

6721 ATGGACATTG TGGACTACCG GTAGTGAGCA CCGTTGATGG ATGTTTGGTT GGAATACATA

6781 GTTTGCCAAA TAATGCGCAA TCCACGAACT ATTATTCAGC CTTCGATGAA GATTTTGAGA

6841 GCAAGTATCT CAGAACTAAT GAACATAATG AATGGATTAA ATCTTGGGTT TATAATCCAG

6901 ATACAGTGTT GTGGGGCCCG CTCAAACTAA AAGAAAGCAC TCCTAAAGGT TTGTTCAAAA

6961 CAACCAAGCT TGTGCAGGAT CTAATTGATC ATGAAGGAGT AGTGGAACAG GCCAAGCACT

7021 CTGCGTGGAT GTATGAAGCC CTGACAGGAA ACTTGCAAGC CGTGGCGACA ATGAAGAGTC

7081 AGCTAGTGAC CAAGCATGTG GTTAAAGGAG AGTGTCGACA CTTTAAAGAG TTCCTAACTG

7141 TGGATGCGGA AGCGGAAGCA TTTTTCAGGC CTTTAATGGA TGCATATGGG AAGAGCTTGC

7201 TGAATAGAGA TGCATATATA AAAGATATAA TGAAGTATTC AAAGCCCATA GATGTTGGAA

7261 TTGTAGATTG TGATGCATTT GAAGAGGCTA TCAATAGAGT TATCATCTAT TTGCAAATGC

7321 ATGGCTTTCA TAAGTGCGCA TACGTAACAG ATGAGCAAGA GATCTTCAAA GCACTCAACA

7381 TGAAAGCTGC CGTTGGAGCT ATGTATGGCG GTAAAAAGAA AGACTACTTT GAGCACTTCA

7441 CTGATGCAGA CAAGGAAGAA ATTGTTATGC AGAGCTGTTT GCGATTGTAC AAAGGCCAGC

7501 TCGGCATTTG GAATGGATCA TTGAAAGCAG AACTTCGGTG TAAGGAGAAA ATACTTGCAA

7561 ATAAAACAAG AACATTCACC GCTGCACCAC TAGACACTCT ACTGGGTGGT AAAGTATGTG

7621 TTGACGACTT CAACAACCAA TTCTACTCGA AGAATATCGA ATGTTGTTGG ACAGTCGGGA

7681 TGACTAAGTT CTATGGTGGC TGGGATAGAC TGCTCCGGCG TTTGCCTGAG AATTGGGTTT

7741 ACTGCGATGC TGACGGCTCA CAATTTGATA GTTCATTGAC TCCATATCTG ATCAATGCTG

7801 TCCTCATCAT TAGAAGCACG TATATGGAAG ATTGGGACGT GGGGTTACAA ATGTTACGTA

7861 ATCTGTACAC GGAGATTGTT TACACCCCCA TATCAACTCC AGATGGAACA ATTGTCAAGA

7921 AGTTTAGAGG GAATAACAGT GGTCAGCCTT CCACTGTAGT GGATAACTCT CTTATGGTTG

7981 TTCTCGCCAT GCACTATGCT CTTATCAAGG AGTGCATCGA TTTTGAAAAG ATTGACAGCA

8041 CGTGCGTGTT CTTTGTAAAT GGCGATGACT TGTTAATTGC TGTAAATCCA GAAAAAGAGA

8101 GTATTCTTGG CAGATTGCAA CAACACTTCT CAGATCTTGG TTTGAATTAT GACTTCTCTT

8161 CAAGAACAAG AAATAAGGAA GAGTTGTGGC TTATGTCCCA TAGAGGTCTG TTGATAGAAG

8221 GCATGTACGT GCCGAAACTT GAGGAAGAAA GAATTGTATC TATCCTACAA TGGGATAGGG

8281 CAGATTTGGC TGAACATAGA CTCGAAGCCA TCTGCGCAGC CATGATAGAG TCCTGGGGCT

8341 ATTCTGAGCT GACTCATCAA ATTAGGAGAT TTTACTCATG GTTGCTGCAA CAACAACCGT

8401 TTGCATCAAT AGCGCAAGAA GGAAAAGCTC CCTACATAGC AAGCATGGCG TTGAGGAAAC

8461 TGTACATGGA TAGGGCAGTG GATGATGAGG AGTTGAAAGC CTTCACTGCA ATGATGGTTG

8521 CATTGGATGA TGAGTTTGAA TGTGATACGT ATGAAGTGCA TCATCAGGCG AATGATACAA

8581 TTGATGCTGG AGGAAGTAGT AAGAAAGATG CAAAATCAGA ACAGAGTAGC ATCCAGCCAA

8641 ATCCTAACAA GGGAAAAGAC AAGGATGTAA ATGTTGGTAC ATCAGGAACA CATACTGTAC

8701 CAAGAATAAA GGCCATTACA TCCAAAATGA GATTGCCCAA AAGCAAGGGA ACAACCGCAC

8761 TAAATTTAGA ACACTTGCTC GAATATGCTC CGCAGCAGAT AGATATCTCA AACACTCGAG

8821 CAACGCAATC ACAGTTTGAC ACGTGGTATG AAGCAGTGCG GGTGGCATAC GACATAGGGG

8881 AAACTGAGAT GCCAACTGTG ATGAATGGGC TTATGGTTTG GTGCATTGAA AATGGAACCT

8941 CGCCAAACAT CAACGGAGTC TGGGTTATGA TGGATGGTGA TGAACAAGTC GAATATCCGT

9001 TGAAACCAAT CGTTGAGAAT GCAAAACCAA CCCTTAGGCA AATCATGGCA CATTTCTCAG

9061 ATGTTGCAGA AGCGTATATA GAAATGCGCA ACAAAAAGGA ACCATATATG CCACGATATG

9121 GTTTAATTCG AAATCTGCGG GATGGAAGTT TAGCGCGCTA TGCCTTTGAC TTTTATGAAG

9181 TTACATCACG AACACCAGTG AGGGCTAGGG AAGCGCACAT TCAGATGAAT GCCGCAGCAT

9241 TGAAATCAGC CCAATCTCGA CTTTTCGGGT TGGATGGTGG CATCAGTACA CAAGAGGAGA

9301 ACACAGAGAG GCACACCACC GAGGATGTCT CTCCAAGTAT GCATACTCTA CTTGGAGTCA

9361 AGAACATGTG ATTGTAGTGT CTCTCCGGAC GATATATAAG TATTTACATA TGCAGTAAGT

9421 ATTTTGGCTT TTCCTGTACT ACTTTTATCA TAATTAATAA TCAGTTTGAA TATTACTAAT

9481 AGATAGAGGT GGCAGGGTGA TTTCGTCATT GTGGTGACTC TATCTGTTGA TTTCGCATTA

9541 TTAAGTTTTA GATAAAAGTG CCGGGTTGTC GTTGTTGTGG ATGATTCATC GATTAGGTGA

9601 TGTTGCGATT CTGTCGTAGC AGTGACTATG TCTGGATCTA TCTGCTTGGG TGGTGTTGTG

9661 ATTTCGTCAT AACAGTGACT GCAAACTTCA ATCAGGAGAC

Appendix C

Genotyping 300 F_1 progeny using ARMS-PCR to identify heterozygous recessive alleles at the *pvr2* locus in pepper.

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
1	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
2	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
3	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
4	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
5	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
6	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
7	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
8	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
9	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
10	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
11	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
12	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
13	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
14	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
15	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
16	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
17	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
18	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
19	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
20	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
21	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
22	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
23	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
24	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
25	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
26	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
27	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
28	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
29	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
30	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
31	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
32	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
33	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
34	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
35	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
36	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
37	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
38	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
39	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
40	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
41	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
42	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
43	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
44	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
45	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
46	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
47	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
48	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
49	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
50	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
51	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
52	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
53	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
54	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
55	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
56	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
57	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
58	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
59	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
60	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
61	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
62	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
63	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
64	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
65	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
66	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
67	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
68	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
69	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
70	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
71	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
72	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
73	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
74	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
75	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
76	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
77	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
78	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
79	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
80	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
81	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
82	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
83	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
84	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
85	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
86	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
87	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
88	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
89	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
90	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
91	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
92	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
93	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
94	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
95	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
96	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
97	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
98	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
99	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
100	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
101	+	+	+	-	+	+	*pvr2+/pvr2*^{1}
102	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
103	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
104	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
105	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
106	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
107	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
108	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
109	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
110	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
111	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
112	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
113	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
114	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
115	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
116	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
117	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
118	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
119	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
120	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
121	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
122	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
123	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
124	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
125	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
126	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
127	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
128	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
129	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
130	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
131	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
132	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
133	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
134	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
135	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
136	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
137	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
138	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
139	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
140	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
141	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
142	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
143	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
144	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
145	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
146	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
147	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
148	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
149	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
150	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
151	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
152	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
153	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
154	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
155	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
156	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
157	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
158	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
159	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
160	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
161	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
162	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
163	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
164	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
165	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
166	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
167	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
168	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
169	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
170	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
171	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
172	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
173	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
174	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
175	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
176	+	+	+	-	+	+	*pvr2+/pvr2*^{1}
177	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
178	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
179	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
180	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
181	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
182	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
183	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
184	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
185	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
186	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
187	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
188	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
189	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
190	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
191	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}
192	+	-	+	+	-	+	*pvr2*^{1}*/pvr2*^{2}
193	+	+	+	-	+	+	*pvr2*$^{+}$*/pvr2*^{1}

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
194	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
195	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
196	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
197	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
198	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
199	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
200	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
201	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
202	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
203	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
204	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
205	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
206	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
207	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
208	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
209	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
210	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
211	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
212	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
213	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
215	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
216	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
217	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
218	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
219	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
220	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
221	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
222	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
223	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
224	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
225	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
226	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
227	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
228	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
229	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
230	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
231	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
232	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
233	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
234	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
235	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
236	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
237	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
238	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
239	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
240	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
241	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
242	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
243	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
244	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
245	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
246	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
247	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
248	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
249	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
250	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
251	+	+	+	-	+	+	*pvr2*+/*pvr2*^{1}
252	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
253	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
254	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
255	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
256	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
257	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
258	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
259	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
260	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
261	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
262	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}
263	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
264	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
265	+	+	+	-	+	+	*pvr2*$^{+}$/*pvr2*^{1}
266	+	-	+	+	-	+	*pvr2*^{1}/*pvr2*^{2}

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
267	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
268	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
269	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
270	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
271	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
272	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
273	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
274	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
275	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
276	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
277	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
278	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
279	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
280	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
281	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
282	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
283	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
284	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$

	Primer						
	T200A		G325A		T236G		
bp	**258**	**199**	**288**	**198**	**176**	**132**	
***pvr2* allele**	**1, 2, 3**	**+**	**+, 1, 3**	**2**	**+, 3**	**1, 2**	
F1 hybrid							**Genotype**
285	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
286	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
287	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
288	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
289	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
290	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
291	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
292	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
293	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
294	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
295	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
296	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
297	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
298	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$
299	+	-	+	+	-	+	$pvr2^{1}/pvr2^{2}$
300	+	+	+	-	+	+	$pvr2^{+}/pvr2^{1}$

Conferences to date

- Moodley, V., Naidoo, R. and Gubba, A. (2013). Development of homozygous pepper (*Capsicum annuum* L.) lines carrying potato virus Y (PVY) resistance genes (*pvr2*^{1} and *pvr2*^{2}) using marker-assisted selection (MAS). 10th International Congress of Plant Pathology, Beijing China. 25 – 30 August.

Publications to date

- Moodley, V., Naidoo, R. and Gubba, A. (2013). Development of homozygous pepper (*Capsicum annuum* L.) lines carrying potato virus Y (PVY) resistance genes (*pvr2*^{1} and *pvr2*^{2}) using marker-assisted selection (MAS). *Acta Phytopathologica Sinica* 43:102.