A PATHOGEN-DERIVED RESISTANCE STRATEGY FOR THE BROAD-SPECTRUM CONTROL OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS INFECTION

by

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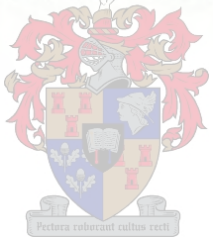

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Grapevine leafroll-associated virus-3 is one of ten members of the *Closteroviridae* that are known to infect grapevine. Nine of these viruses are associated with grapevine leafroll disease, of which GLRaV-1 and GLRaV-3 are the most important and widespread. Members of the *Closteroviridae* are unique amongst the viruses, as it is the only known family whose members encode a heat shock protein 70 kDa homolog (Hsp70h). The Hsp70h is a movement protein (MP) that is required for the active translocation of the virion structure through the plasmodesmata into adjacent cells. Broad-spectrum resistance to unrelated viruses can be obtained by a pathogen-derived resistance (PDR) strategy that is based on the expression of a dysfunctional MP in plants. The Hsp70h has two distinct domains. The N-terminal two thirds of the protein is an ATPase domain and shares high homology with the ATPase domains of all Hsp70h proteins from the *Closteroviridae* and Hsp70 proteins from the prokaryote and eukaryote kingdoms. Conserved amino acids are found in the ATPase domain and are required for the positioning of the ATP at the catalytic site for ATP hydrolysis. The C-terminal domain is variable and the function of this domain in the *Closteroviridae* is not known. In prokaryote and eukaryote Hsp70 proteins, the C-terminal domain is required for protein-protein interactions.

The American NY-1 isolate of GLRaV-3 has been sequenced and PDR strategies have been attempted with the coat protein, divergent coat protein and replicase genes, but not with a dysfunctional form of the *hsp70h* gene. In this study, double-stranded RNA was isolated from a commercial vineyard with unknown virus status, but with distinct grapevine leafroll symptoms, and from two grapevine sources of known virus status, one with mild and one with severe symptoms. The GLRaV-3 *hsp70h* gene was amplified by RT-PCR from the dsRNA and the gene sequence was analysed. The *hsp70h* gene from the three virus sources contained more than 94% nucleotide sequence homology to the NY-1 isolate and the conserved amino acids required for ATPase activity were present. The *hsp70h* gene isolated from GLRaV-3 from a commercial Stellenbosch vineyard showing clear leafroll symptoms was selected for further work and was subjected to site-directed mutagenesis to engineer four point mutations in the gene. These four mutations resulted in the substitution of Asn for Asp^{6}, Gly for Thr^{10}, Lys for Glu^{174} and Asn for Asp^{197}.

The wild type (WT) and mutated (Mut) forms of the *hsp70h* genes were cloned into a bacterial expression vector. Expression of both the WT- and Mut-Hsp proteins was achieved, and the protein was expressed in the insoluble inclusion bodies. All attempts to refold and isolate active proteins from the inclusion bodies were unsuccessful. Attempts to increase the concentration of soluble protein within the expressing bacteria were unsuccessful. Due to the lack of active protein, biochemical tests on the ATPase activity of the WT- and Mut-Hsp proteins could not be conducted.

The *wt*- and *mut-hsp* genes were cloned into a plant expression vector for transformation into tobacco plants. These transformations were successful and gave rise to 22 Km^r and 18 Km^r plants from the WT- and Mut-Hsp constructs respectively. Two plant lines, M5 and M10, transformed with the *mut-hsp* transgene construct, appeared to have a high level of resistance to the challenging *potato X potexvirus*, whereas all the other tested plants were susceptible to the challenging virus. It was thus shown that a dysfunctional form of the GLRaV-3 Hsp70h could provide resistance to an unrelated virus in tobacco.

OPSOMMING

Wingerdrolblaar-geassosieerde virus 3 (GLRaV-3) is een van 10 lede van die *Closteroviridae* wat wingerd kan infekteer. Nege van die virusse is met wingerdrolblaar geassosieer. Die GLRaV-1 en GLRaV-3 is die belangrikste en mees wyd verspreide lede van die rolblaar-geassosieerde *Closteroviridae*. Lede van die *Closteroviridae* is uniek in die opsig dat die virusse vir 'n 70 kDa-homoloë hitteresponsproteïen (Hsp70h) kodeer. Die Hsp70 is 'n bewegingsproteïen (MP) wat belangrik is vir die translokasie van die virus deur die plasmodesmata na die naasliggende sel. Breë-spektrum weerstand teen onverwante virusse kan behaal word deur 'n patogeen-afgeleide weerstandstrategie (PDR), wat op die uitdrukking van 'n disfunksionele MP wat in plante uitgedruk word, gebaseer is. Die Hsp70h-proteïen het twee gebiede. Die N-terminale gebied is 'n ATPase-gebied en toon hoë homologie met ander ATPase-gebiede van Hsp70h-proteïene van die *Closteroviridae*, asook die prokariotiese en eukariotiese koninkryke. Gekonserveerde aminosure wat belangrik is vir die posisionering van ATP in die katalitiese domein vir ATP-hidrolise is in die ATPase-gebied gevind. Die C-terminale gebied is variërend en die funksie van die gebied in die *Closteroviridae* is onbekend. In prokariotiese en eukariotiese Hsp70h-proteïene is die C-terminale gebied belangrik vir proteïen-proteïen interaksies.

Die nukleotiedvolgorde van die Amerikaanse NY-1-isolaat van GLRaV-3 is al bepaal en PDR-strategieë is ook op die kapsiedproteïen, uiteenlopende kapsiedproteïen en die replikasie-proteïen uitgevoer, maar nog nie op 'n disfunksionele vorm van die Hsp70h-geen nie. In hierdie studie is dubbelstring-RNA (dsRNA) van 'n kommersiële wingerd met onbekende virusstatus wat rolblaarsimptome toon, geïsoleer, asook van twee wingerde met 'n bekende virusstatus, een met ligte en een met strawwe simptome. Die GLRaV-3 *hsp70h*-geen is met hulp van die polimerasekettingreaksie-metode (PKR) vanaf die dsRNA geamplifiseer en die geen se nukleotiedvolgorde is bepaal. Die *hsp70*-gene van drie verskillende wingerde het meer as 94% homologie met die NY-1-isolaat getoon. Die gekonserveerde aminosure wat vir ATPase-aktiwiteit belangrik is, was teenwoordig. Die *hsp70h*-geen van GLRaV-3, wat uit 'n kommersiële wingerd met duidelike rolblaarsimptome in die Stellenbosch-gebied geïsoleer is, is vir verdere navorsing gekies en dit is aan setel-gerigte mutagenese blootgestel om vier mutasies van die geen te bewerkstellig. Die gevolg van hierdie vier mutasies was die verandering van Asn na Asp^{6}, Gly na Thr^{10}, Lys na Glu^{174} en Asn na Asp^{197}.

Die wilde (WT) en veranderde (Mut) vorms van die *hsp*-gene is in 'n bakteriese uitdrukkingsvektor gekloneer. Uitdrukking van beide die WT- en die Mut-Hsp-proteïene is behaal, maar die proteïene was in die onoplosbare fraksie geleë. Pogings om die onoplosbare proteïene te isoleer en in 'n aktiewe oplosbare vorm te verkry, was onsuksesvol. Verdere pogings om die proteïene in die oplosbare fraksie van die bakteriese ekspressiesisteem uit te druk, was ook onsuksesvol. As gevolg

van die gebrek aan aktiewe proteïen kon biochemiese toetse nie op die ATPase-aktiwiteit van die WT- en Mut-Hsp proteïne gedoen word nie.

Die *wt*- en *mut-hsp*-gene is ook in 'n plantekspressievektor gekloneer vir transformasie in tabakplante. Hierdie transformasies was suksesvol en het aanleiding gegee tot 22 kanamisienbestande (Km^r) en 18 Km^r plante vanaf die WT- en Mut-Hsp-konstrukte onderskeidelik. Twee plantlyne, M5 en M10, wat met die *mut-hsp*-transgene getransformeer is, het 'n hoë vlak van weerstand teen die infekterende aartappelvirus X getoon in vergelyking met ander plante wat met die virus geïnfekteer is. Daar is dus bewys gelewer dat 'n disfunksionele vorm van die GLRaV-3 Hsp70h weerstand kan bied teen 'n onverwante virus in tabak.

This dissertation is dedicated to:
M. Barbara von Wechmar

BIOGRAPHICAL SKETCH

Michael-John was born in the Cape Province in 1971. He matriculated from The Settlers High School in 1989, after which he attended the University of Cape Town, where he completed a Bachelor of Science (Microbiology) in 1992. He completed a Bachelor of Science (Honours) (Microbiology) degree in 1993, following which he worked as research technician in the Department of Microbiology at the University of Cape Town for two years. He completed a Master of Science degree in Molecular Virology in 1996, after which he moved to the University of Stellenbosch to take up a position as research technician at the Institute for Wine Biotechnology. He registered for a PhD in Wine Biotechnology in 1998.

Michael-John married Andrea in 1999, and they are expecting their first child in August 2003.

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PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced and referenced separately.
Chapter 2 will be submitted in its entirety to the *South African Journal of Enology and Viticulture* as a review paper.
Chapter 3 will be submitted to *Virus Genes* as a short communication focusing on the phylogenetic relationships of the *Closteroviridae*.

Chapter 1 **General Introduction and Project Aims**

Literature Review

Chapter 2 **Grapevine leafroll disease: what is it, can it be controlled?**

Research Results

Chapter 3 **Polymerase chain reaction amplification of the *grapevine leafroll-associated virus-3 hsp70h* gene from various grapevine sources**

Chapter 4 **The mutagenesis and bacterial expression of the Hsp70h from *grapevine leafroll-associated virus-3***

Chapter 5 **A dysfunctional *grapevine leafroll-associated virus-3* Hsp70h protein confers resistance against the unrelated *potato X potexvirus* in transgenic tobacco**

Chapter 6 **General discussion and conclusions**

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Plants are the primary producers of food in the ecosystem and most life depends on them. Their health is of vital importance to mankind. The growth and productivity of plants determines the food supply of all animals, including man. Plant diseases and pests affect plant productivity and therefore the supply of food to animals and man. Unlike natural disasters, such as floods and droughts, plant diseases and pests can be effectively managed and their impact on plant production can be reduced. This is particularly true in the developed economies of the Western World. As the world population continues to increase, the demand on the food supply will be evermore stretched. To overcome this dilemma, science needs to assist agriculture to develop strategies that can protect food crops and enhance productivity without necessarily increasing acreage under cultivation. The obvious route to protect and improve plant productivity is to increase resistance to and tolerance of diseases and pests.

Plant viruses can cause significant crop losses in agriculturally important crop plants. They account for a considerable portion of the losses suffered annually from crop diseases and losses have been estimated at US$60 billion per year (Cann, 1997). Viruses cause disease by utilising cellular machinery for their own replication, thereby disrupting cellular processes. These disruptions interfere with cellular metabolism and lead to the development of abnormal substances, which interfere with the life of the plant and can affect crop yield and quality (Cann, 1997). Plant viruses that cause significant crop losses are the focus of research programmes designed to control these viruses.

In order for a plant virus to systemically infect a plant and cause a disease and subsequent crop loss, it must be deposited in a plant cell through mechanical damage, vector transmission or plant propagation. Virus infection continues with the replication of the genome, movement from the site of infection to the vascular tissue and subsequent movement throughout the remainder of the plant (Mathews, 1991).

1.2 CONTROL OF PLANT VIRUSES

A major problem with the control of plant viruses is the ecology of viruses. Plant viruses are vectored by a variety of invertebrate vectors that are themselves difficult to control. The involvement of humans exacerbates the problem, due to interference in the natural ecology of insects, the spread of viruses by plant propagation and the formation of large tracts of monoculture crops. Plant sanitation, quarantine and certification can be successfully instituted for the control of plant viruses; however, where viruses are endemic, these strategies do not deliver good results. Classical approaches for the control of viruses, based on conventional breeding, are effective, but take too long, are costly and resistance can be overcome by virus variation. A

further problem arises because certain resistance genes cannot be incorporated into breeding programmes because of barriers to sexual hybridisation between the crop plant and wild varieties that contain resistance (Kavanagh and Spillane, 1995; Lomonossoff, 1995). Engineered protection utilising molecular techniques can be achieved by the incorporation of pathogenesis-related proteins, antiviral proteins, natural host resistance genes and plantibodies (Kavanagh and Spillane, 1995). Virus strain variation may, however, overcome some of these approaches.

A further approach for virus control in plants was suggested by Sanford and Johnson (1985). This approach was termed pathogen-derived resistance (PDR). In this approach, genes derived from the pathogen, are expressed in the plant at an inappropriate concentration or form or at an inappropriate time in the infection cycle, interfering with the virus infection and reducing the pathogenicity of the virus. The first example of this approach was achieved in 1986, when Powell-Abel *et al.* (1986) obtained resistance to *tobacco mosaic tobamovirus* (TMV) in transgenic plants expressing the TMV coat protein (CP). Since this pioneering experiment, more than 30 viruses have been targeted for control through PDR. Any coding or non-coding sequence of the viral genome can be used for PDR. The most common approach is the use of the CP, but any of the replication (Rep)-associated proteins, the movement protein (MP) or any other non-structural protein can also be used (Lomonossoff, 1995). Coat protein-mediated resistance (CPMR) and replicase-mediated resistance (RepMR) offer very high levels of resistance to homologous viruses, or viruses that are very closely related but do not offer resistance against unrelated viruses (Kavanagh and Spillane, 1995).

Pathogen-derived resistance can be achieved either through the accumulation of RNA or through the production of proteins (Baulcombe, 1996). RNA-dependent mechanisms exhibit low mRNA accumulation levels and function through the sequence-specific RNA degradation. This results in the prevention of the accumulation of RNA that is homologous to the transgene (Baulcombe, 1999). This RNA-dependant resistance results in very high levels of resistance, however this resistance is very specific to the targeted gene. Protein-dependent mechanisms exhibit high levels of mRNA expression and the mechanism of resistance varies depending on the target. In general, the expressed protein interferes with the infection cycle and inhibits a particular process from occurring. An example is the TMV CP, which is thought to prevent the uncoating of the RNA and thus prevents viral replication (Register and Beachy, 1988).

The coat protein, movement protein and replicase genes have been used for the control of a variety of plant viruses, including *grapevine fanleaf nepovirus* (Barbier *et al.*, 2000; Gölles *et al.*, 2000; Krastanova *et al.*, 2000), *grapevine A vitivirus*, *grapevine B vitivirus* (Gölles *et al.*, 2000; Martinelli *et al.*, 2000), GLRaV-2 and GLRaV-3 (Krastanova *et al.*, 2000). The coat protein genes from GLRaV-2 and GLRaV-3 have also been incorporated into the grapevine genome (Krastanova *et al.*, 2000). Results on the effectiveness of this coat protein-mediated resistance for the

control of leafroll disease are still not available. Genetic engineering shows a great deal of promise for the control and management of leafroll disease and further research is warranted. However, the use of genetic manipulation of grapevines to increase resistance against leafroll disease should be used in conjunction with other standard techniques, such as the supply of "virus-free" material, general sanitation, vector control strategies and viticultural management strategies.

1.2.1 MOVEMENT PROTEIN-MEDIATED RESISTANCE

Although plant viruses appear rather simple, containing between three and 16 genes, they are very diverse and use a variety of different strategies for spread to plants, entry into cells and replication within cells. Their infection process is further complicated by interactions with the host's cellular machinery, which it uses to maintain infection. However, the process of cell-to-cell movement within the plant is similar amongst most plant viruses. Virus movement within a plant requires a viral-encoded MP that interacts with the cellular plasmodesmata to facilitate the movement of the virus through these intercellular channels. PDR strategies based on interference with virus movement have proven to be successful for the broad-spectrum control of viruses.

Movement protein-mediated resistance (MPMR) can provide broad-spectrum resistance when the mechanism of action is based on the expression of a dysfunctional MP (Beck *et al.*, 1994; Cooper *et al.,* 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.*, 1997; Tacke *et al.*, 1996). Resistance is achieved when the dysfunctional MP, expressed at high levels, out-competes the functional virus encoded MP, thus preventing the virus from undergoing movement to adjacent cells and tissues (Beck *et al.*, 1994; Cooper *et al.,* 1995; Lapidot *et al.*, 1993). The levels of resistance obtained by MPMR are much lower than those observed by CPMR or RepMR (Kavanagh and Spillane, 1995; Lomonossoff, 1995).

A greater understanding of the function of the MP and their interaction with cellular components will lead to improved knowledge on the domains and amino acids specifically required for virus movement. With this knowledge, mutagenesis of essential domains of the MP could be performed, which could in turn be used to engineer more effective broad-spectrum resistance against viral pathogens through the expression of a single dysfunctional gene.

1.3 VIRUS MOVEMENT

The movement of viruses is an important part of viral pathogenesis. Viruses must move from the site of infection to adjacent plant cells and then to other organs via the vascular system. If viruses are unable to move to adjacent cells and organs, a subliminal infection develops in which the plant does not appear to be infected, but appears to be immune to the viral disease. If, however, the virus is able to move to

adjacent cells and organs, a progressive infection develops, resulting in a systemic infection.

The major barrier to plant virus movement comes in the form of the impermeable cell wall, which acts as a barrier to the release and uptake of progeny viruses by surface fusion and receptor-mediated endocytosis, which are mechanisms used by animal viruses (Cann, 1997). Because the plant cell wall acts as a barrier to the extracellular release and uptake of plant virus progeny, plant viruses have evolved a mechanism for cell-to-cell movement that evades the cell wall barrier (Atabekov and Dorokhov, 1984). Plant viruses encode a MP that interacts with the plasmodesmata and facilitates the movement of the virus into the adjacent cells. Plant viruses move from cell-to-cell and over long distances by modifying the pre-existing pathways for macromolecular transport provided by the plasmodesmata and vascular tissue (Atabekov and Taliansky, 1990).

The plasmodesmata are important for the movement of water, nutrients and macromolecules between adjacent cells. They act as an intercellular channel that maintains a continuum between the plant cells. The plasmodesmata are lined with the endoplasmic reticulum, which is contiguous to both the adjoining cells. Extending through the plasmodesmata is a desmotubule, which links the endomembrane of the adjoining cells. Some plasmodesmata have a central cavity between the desmotubule and the plasma membrane. Plant viruses are thought to exploit this space for movement through the plasmodesmata (Pennazio *et al.,* 1999). The plasmodesmal channels are too small to allow virus particles or nucleoprotein particles to move through. The plant virus MPs modify the plasmodesmata and facilitate the movement of the virus into adjacent cells.

Two mechanisms for cell-to-cell movement have been determined. Both require the modification of the plasmodesmata by the virus-encoded MP. First, *tobacco mosaic tobamovirus* (TMV)-like mechanism, whereby the virus moves in a non-virion form through the plasmodesmata (Citovsky and Zambryski, 1991). Second, *cowpea mosaic comovirus* (CPMV)-like mechanism, whereby the virus particles moves through a tubule structure to adjacent cells (van Lent *et al.*, 1990). Furthermore, viruses have different protein requirements for cell-to-cell movement in the plant. The TMV 30 kDa MP binds to viral RNA to form a MP-RNA nucleoprotein complex, which is targeted to the plasmodesmata. The TMV MP also acts to increase the size exclusion limit of the plasmodesmata to allow the large nucleoprotein complex to move through.

The movement of viruses in a host plant is an active process divided into two distinct phases, viz. short distance cell-to-cell movement and long distance vascular movement. The cell-to-cell movement is slow (μm per hour) and the long distance movement through the vascular tissue is fast (cm per hour). Cell-to-cell movement is an active process that is facilitated by the MP in combination with a number of other proteins, including the CP and Rep proteins. The long distance movement of plant viruses is less well understood; it is a “passive” process that appears to follow the

normal source-to-sink flow of photo-assimilates. Long distance movement appears to require the MP, CP and Rep proteins, although these proteins are not required for the active translocation of the virus through the vascular tissue. Long distance movement of most viruses requires that the virion be formed to protect the viral genome. The role of the CP in long distance movement is unknown, but it may help the MP to dilate the plasmodesmata and increase permeability of the plasmodesmata at the boundary between the vascular and non-vascular tissue (Citovsky and Zambryski, 2000).

The movement of viruses in systemically infected plants is divided into four phases. The virus must move from the site of infection by cell-to-cell movement through the epidermal and parenchyma cells. It must then move from the parenchyma through the sieve elements, bundle sheath cells, phloem parenchyma and companion cells into the vascular tissue. It then undergoes long distance bulk flow through the vascular tissue. Finally, the virus moves from the vascular tissue into the phloem parenchyma cells for cell-to-cell movement back to the epidermal cells (Atabekov and Taliansky, 1990). Viruses that are limited to the phloem tissue have limited cell-to-cell movement, but have efficient long distance movement mechanisms.

The movement of viruses is complex and consists of active cell-to-cell movement and "passive" long distance movement. However, all viruses need to modify the plasmodesmata in order to facilitate the movement of viruses to adjacent cells. This process appears to be conserved amongst the plant viruses and is achieved by the viral-encoded MP. For efficient pathogenesis and a systemic infection, a plant virus must undergo both forms of movement in order to infect the plant. By blocking the movement of viruses in the plant, a subliminal infection may develop, resulting in control of the virus infection.

1.4 GRAPEVINE LEAFROLL DISEASE

Virus diseases of the perennial grapevine were originally classified as transmissible diseases for which no pathogen could be isolated. These diseases were mostly transmitted by plant propagation and the grafting of an infected bud onto a healthy plant (Goheen, 1988). Grapevine leafroll disease is one of the three most important viral diseases of grapevine (Martin *et al.*, 2000) and is found in all countries in which grapevines are grown (Goheen, 1988). Grapevine leafroll disease causes a significant loss in fruit quantity and a reduction in fruit quality (Goheen, 1988). Nine different members of the *Closteroviridae* are associated with the grapevine leafroll disease. Of these nine viruses, *grapevine leafroll-associated virus-1* (GLRaV-1) and *grapevine leafroll-associated virus-3* (GLRaV-3) are known to cause grapevine leafroll disease (Boscia *et al.*, 1995). The seven remaining grapevine-infecting *Closteroviridae* members are associated with leafroll, but have only been detected in mixed infections with either GLRaV-1 or GLRaV-3 and their role in the disease is still

unknown. The GLRaVs are 1400-2000 nm in length and have been poorly characterised because of the difficulty of virion purification, low virus titres in infected plant material, the tendency for mixed infections and the lack of alternate host species. The GLRaVs are phloem limited, graft transmissible and can spread in the field due to insect vector transmission. *Grapevine leafroll-associated virus-1* and GLRaV-3 are transmitted by mealybug and scale insects (Belli *et al.*, 1994, Golino *et al.*, 1995). Insect transmissibility of the seven remaining leafroll-associated *Closteroviridae* is unknown. Many of the grapevine-infecting *Closteroviridae* members also appear to be associated with other diseases, including stem-pitting and shiraz decline. Nucleotide sequencing of the genome of the grapevine-infecting *Closteroviridae* members is facilitated by the ease with which the double stranded replicative form RNA (dsRNA) can be purified from the stem cortex of infected grapevines. This plant tissue can be used to overcome the difficulties observed with the purification of intact virion particles and can be exploited for gene cloning.

Grapevine leafroll-associated virus-3 is widespread and has been consistently found in South African grapevines showing leafroll symptoms (Engelbrecht and Kasdorf, 1990; Nel and Engelbrecht, 1972). The prevalence of grapevine leafroll, the consistent detection of GLRaV-3 and the vector transmissibility of GLRaV-3 suggest that it may be the major virus associated with grapevine leafroll disease in South Africa.

1.5 PROJECT PROPOSAL

This project forms part of a wider programme to enhance disease resistance against plant viruses in grapevines. The aims of the programme are to incorporate genes that can provide resistance against GLRaV-3 in particular, as well as other grapevine-infecting viruses. For the latter, a broad-spectrum-resistance strategy was planned. This particular project was important to prove the concept that a dysfunctional MP from GLRaV-3 could be used to provide resistance against an unrelated plant-infecting virus. To prove the concept of resistance, the dysfunctional MP transgene was transformed into the model system presented by tobacco plants. These plants, containing the dysfunctional MP, would be challenged by an unrelated virus and the levels of virus accumulation and rates of virus movement would be determined. To satisfy the criteria for a challenging virus, *potato x potexvirus* (PVX) was selected. This choice was based on the following criteria: 1) PVX is unrelated to GLRaV-3; 2) PVX is able to systemically infect tobacco plants; 3) PVX has been successfully maintained in our laboratory and 4) specific and reliable detection assays are available for PVX. The homologous GLRaV-3 was not used for resistance experiments, due to the fact that GLRaV-3 can not be inoculated to tobacco plant, mechanically or otherwise. Furthermore, resistance against the homologous virus could be due to either an RNA- or a protein-mediated mechanism (Tacke *et al.*, 1996). RNA-mediated resistance is well known to be effective only against the

homologous virus. Hence, to obtain broad-spectrum resistance, a protein-based mechanism is required and testing with an unrelated virus would provide evidence for a protein based resistance mechanism.

The results obtained from this project would in turn provide the basis of further work, which would prove broad-spectrum resistance to the homologous GLRaV-3 and other unrelated viruses, either in tobacco and / or in grapevine. To prove the concept that a dysfunctional form of the GLRaV-3 MP could provide resistance in transgenic plants against the unrelated PVX challenge virus, the following experimental steps were taken:

1. Identification of GLRaV-3 infected grapevine material and the isolation of dsRNA for further molecular work;
2. Amplification of the *hsp70h* gene from GLRaV-3;
3. Synthesis of a dysfunctional copy of the Hsp70h protein by the site-directed mutation of conserved amino acids required for the correct functioning of the ATPase domain;
4. Transformation of these constructs into tobacco cells and the selection of transgenic plants that express the transgenes;
5. Challenging the transgenic plants with the unrelated PVX.

The focus of this project was on GLRaV-3, because this virus is known to cause grapevine leafroll disease and is widespread in South African vineyards. The *hsp70h* gene was targeted for a PDR strategy, because this protein has been shown to be integrally involved in movement of the *beet yellows closterovirus.* Pathogen-derived resistance strategies based on the movement protein have been shown to provide broad-spectrum resistance to unrelated viruses. In the case of grapevine leafroll, and in grapevines in general, many viruses are associated with virus-related diseases. Therefore, a successful MPMR strategy might be successful in controlling all nine of the *Closteroviridae* members associated with grapevine leafroll disease.

With the confirmation of the concept that a mutated form of the GLRaV-3 *hsp70h* gene can confer resistance to plants against challenge by an unrelated virus, the dysfunctional Hsp70h construct can be transformed into grapevine callus for further testing against grapevine-infecting *Closteroviridae* members. Members of the *Closteroviridae* are not easily transmitted mechanically, and members of the *closterovirus* genus can be transmitted to tobacco hosts only with great difficulty (Martelli *et al.*, 2002). Therefore, in order to test for resistance against GLRaV-3 and the other grapevine-infecting members of the *Closteroviridae*, the construct must be transformed into easily transformed grapevine cultivars, such as Sultana. Following this, the construct should also be transformed into commercial wine grape cultivars to allow for the commercial release of the virus-resistant material. This work will only be applicable once we have been able to prove that plants containing a dysfunctional copy of the GLRaV-3 *hsp70h* are resistant to infection when challenged with an unrelated virus. We will also need to prove that the observed resistance against viruses is observed in grapevine material.

The benefits to the South African grape and wine industry of reduced leafroll disease could be very significant.

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CHAPTER 2

LITERATURE REVIEW

Grapevine leafroll disease: what is it, can it be controlled?

GRAPEVINE LEAFROLL DISEASE: WHAT IS IT, CAN IT BE CONTROLLED?

2.1 INTRODUCTION

Forty-nine viruses are reported to infect grapevine (Alkowni *et al.* 2002; Martelli, 1997; Monis and Bestwick, 1997). This number is bound to increase as detection techniques become more sensitive. The three most important virus disease complexes of grapevine are; degeneration and decline, caused by members of the *Comoviridae*; rugose wood, caused by the *vitiviruses*; and leafroll, caused by members of the *Closteroviridae* (Martin *et al.*, 2000).

Grapevine leafroll has been known as rougeau, flavescence, brunisure, Rolkrankheit, red leaf and white emperor disease. First reported in France in the 1850s, grapevine leafroll disease now has a worldwide distribution. The increasing incidence of grapevine leafroll disease poses a serious problem to both the grape and wine industries. The disease is graft transmissible and has been spread predominantly through the movement of infected planting material (Goheen, 1988), but can also spread through transmission by mealybug and scale insect vectors.

A number of different viral pathogens have been found to be associated with grapevine leafroll disease. These viruses include nine *Closteroviridae* members, *nepoviruses*, *vitiviruses* and the unassigned grapevine fleck virus. The viruses associated with grapevine leafroll disease are limited to the phloem tissue, particularly in the canes and leaves, with the older basal leaves showing distinct leafroll symptoms. Leafroll symptoms are expressed in mid- to late-summer and include the rolling down of the leaf blade, brittleness of the leaf, interveinal chlorosis or interveinal reddening with dark green veins, delayed fruit maturation and lighter coloured fruit from red cultivars. Severely infected vines may also show symptoms of dwarfing or poor vigour. Grapevine leafroll disease has a negative effect on the quantity and quality of the yield of infected vines. Yield losses of up to 20% have been reported (Goheen, 1988), but wine quality losses have not been quantified.

The complex nature of grapevine leafroll disease will be explored in this review. The virus agents that have been associated with grapevine leafroll disease will be discussed, and their subsequent effect on grapevine physiology and fruit quality. Focus on the worldwide incidence of grapevine leafroll disease, the vectors that cause its spread within the vineyard and aspects of leafroll detection will also be discussed. Finally, possible methods that may be used to prevent infection, control and manage this disease and reduce the negative effects on fruit quality, will be discussed.

2.2 GRAPEVINE LEAFROLL DISEASE

Unfavourable environmental conditions, scale insects, myxomycetes, protozoa, fungi and nutrient deficiencies have all been blamed for grapevine leafroll disease (Hoefert and Gifford, 1967a). Scheu (1936) first determined the viral nature of grapevine leafroll disease by graft transmission from diseased to healthy vines. The identification of viral pathogens from leafroll-infected vines has, however, been difficult due to the lack of mechanical transmission from infected vines. Mechanical transmission of grapevine leafroll has been reported by Goszczynski *et al.* (1996) and is most likely due to transmission of the GLRaV-2 *closterovirus*.

2.2.1 VIRUSES ASSOCIATED WITH GRAPEVINE LEAFROLL DISEASE

Numerous viruses have been detected in leafroll-infected grapevines. These include members of the *Closteroviridae* (Castellano *et. al.*, 1983; Choueiri *et al.* 1996; Faoro *et al.,* 1981; Hu *et al.*, 1990a; Hu *et al.*, 1990b; Monis, 2000; Mossop *et al.*, 1985; Namba *et al.*, 1979; Zee *et al.*, 1987), isometric viruses (Castellano *et al.*, 1983; Engelbrecht and Kasdorf 1990a), *potyviruses* (Tanne *et al.*, 1974) and *vitiviruses* (Agran *et al.*, 1990; Boscia *et al.*, 1990; Milne *et al.*, 1984). The diversity of viruses found in leafroll-infected grapevines may be due to the vegetative propagation of grapevines and the ability of the perennial grapevine to accumulate multiple viruses over numerous years of cultivation (Karasev 2000). The vegetative propagation of grapevine can transmit viruses to progeny vines. When grafted onto rootstock material that contains other viruses, progeny scion material may subsequently lead to complex virus infections, which are often found in grapevines. Another explanation for the complex nature of grapevine leafroll disease may be that grapevines were often cultivated in the presence of other crops before they were grown in monoculture, thus allowing viruses to spread into the grapevine from other hosts.

Isometric particles with a diameter of 22 to 24 nm were detected by electron microscopy in the sieve elements and phloem tissues in 23 of 47 leafroll-infected grapevine samples (Castellano *et al.*, 1983). These virus particles were found scattered throughout the cytoplasm or in the central vacuole. Virus particles were found to be distributed randomly, in a crystal structure or in a tubular structure, as seen with the *Nepoviridae* and *Comoviridae*. Five samples that were mechanically transmitted were infected with *grapevine fanleaf nepovirus* (GFLV) and two virus samples were identified as isometric virus particles other than GFLV (Castellano *et al.*, 1983). Engelbrecht and Kasdorf (1990a) found similar isometric virus particles in a vine, but at very low titres. Subsequent transmission studies induced typical grapevine fleck symptoms in *Vitis rupestris*. These results indicate that GFLV and grapevine fleck virus can be found in leafroll-infected vines. These viruses, however, are not regarded as causative agents of grapevine leafroll disease.

Potyvirus particles were mechanically transmitted to herbaceous hosts from leafroll-infected vines (Tanne *et al.*, 1974) and isolated from these infected

herbaceous host plants (Tanne *et al.*, 1977). *Potyviruses*, however, were not detected in leafroll-infected grapevines (Tanne *et al.*, 1977). Unconfirmed reports indicate that Shalla and Goheen (as reported in Tanne *et al.,* 1977) found similar potyvirus-like particles in one grapevine sample. There are no other reports of *potyvirus* infection in grapevine. Current data suggest that *potyviruses* are not responsible for grapevine leafroll disease.

Classified originally as closteroviruses in the early literature, *vitiviruses* are long flexuous particles of approximately 800 nm in length. *Vitiviruses* are associated with the rugose wood disease complex (Conti *et al.*, 1980; Martelli, 1997), but are routinely detected in leafroll-infected grapevines (Agran *et al.*, 1990; Credi and Giunchedi, 1996; Digiaro *et al.* 1994; Goszczynski and Jooste, 2003; Guidoni *et al.*, 1997). *Vitiviruses* are not the causative agent of grapevine leafroll disease. In South Africa, grapevine A vitivirus (GVA) has been shown to have a very close association with grapevine leafroll-infected grapevines (Goszczynski and Jooste, 2003).

The *Closteroviridae* contain viruses with long flexuous particles with two modal lengths. Short particles are 650-900 nm and long particles are 1200-2200 nm in length. Long particles have routinely been shown to be associated with grapevine leafroll (Castellano *et al.*, 1983; Faoro *et al.*, 1981; Hu *et al.*, 1990a; Mossop *et al.*, 1985; Namba *et al.*, 1979; Zee *et al.*, 1987). Members of the *Closteroviridae* are consistently isolated and detected in leafroll-infected vines (Tzeng *et al.*, 1999) and are currently thought to be the causative agent responsible for grapevine leafroll disease (Martelli, 1993; 1997). The association of *Closteroviridae* members with grapevine leafroll disease is further confirmed by studies with heat-treated grapevines. Leafroll antigens, which are present in primary infected grapevines, were not detected in grapevines subjected to heat treatment (Guidoni *et al.*, 1997; Zimmermann *et al.*, 1990) or meristem tissue culture (Monis, 2000). These techniques are used to eliminate viruses from plant material. These observations suggest that members of the *Closteroviridae* are responsible for grapevine leafroll disease. This, however, needs to be further proven by re-infection of these virus isolates into healthy virus-free vines and the subsequent development of typical grapevine leafroll symptoms. Such experiments have not been reported to date.

2.2.1.1 The *Closteroviridae* associated with grapevine leafroll disease

The *Closteroviridae* comprise filamentous plant viruses transmitted by insect vectors. These viruses produce characteristic cytopathological effects in infected cells and have a large positive-strand ssRNA genome. The *Closteroviridae* represent a group of emerging and re-emerging, economically important plant pathogens (Karasev, 2000).

Grapevine leafroll-infected vines seldom contain single virus isolates (Hu *et al.*, 1990a; Monis and Bestwick, 1997; Zimmermann *et al.*, 1990), but mixed infections are common (Boscia *et al.*, 1995; Hu *et al.*, 1990a; Monis and Bestwick, 1996). Hu *et al.* (1990a) reported that some leafroll-infected grapevines might contain three

serologically distinct members of the *Closteroviridae*. Although it is conceivable that leafroll-infected vines may contain more than three leafroll-associated viruses, there have been no reports of this to date.

Grapevine leafroll-associated virus-1, GLRaV-3, GLRaV-4 and GLRaV-5 consist of a single virion particle of between 1400-2200 nm in length and contain a single positive-sense ssRNA of 16.9 –19.5 kb in size (Martelli *et al.,* 2002). *Grapevine leafroll-associated virus-2* consists of a single virion particle of 1800 nm and contains a single positive-sense ssRNA of the order of 15.5 kb. As with other members of the *closterovirus* genus, it is mechanically transmissible to tobacco plants (Martelli *et al.*, 2002).

Grapevine leafroll-associated virus-1 and GLRaV-3 are the most widespread of the grapevine-infecting *Closteroviridae*. They are consistently associated with grapevine leafroll symptoms and are genuine causal agents of grapevine leafroll disease (Belli, 1995; Boscia *et al.*, 1995). *Grapevine leafroll-associated virus-2*, although associated with grapevine leafroll disease, is less commonly detected and is associated with a rootstock–scion incompatibility disorder (Boscia *et al.*, 1995; Greif *et al.*, 1995; Monis and Bestwick, 1997). The association of the six remaining *Closteroviridae* members with grapevine leafroll disease is based on circumstantial evidence. These viruses have not been proven to cause grapevine leafroll symptoms in single infections (Alkowni *et al*, 2002; Boscia *et al.*, 1995; Choueiri *et al.*, 1996; Monis and Bestwick, 1997).

There is very little information available on the physicochemical properties of these viruses (Table 2.1). This is due to the lack of mechanical transmission, the lack of alternate host plant species, the low yields of purified virus particles and the high incidence of mixed infections. Limited virus purification studies (Uyemoto *et al.*, 1997) indicate that GLRaV-3 is a stable virus isolate. The virus is stable for a period of up to three months when stored at either 4°C or -20°C (Hu *et al.*, 1990a). The stability of the other *Closteroviridae* members associated with leafroll is not known. The buoyant density of members of the *Closteroviridae* is dependent on the length of the virus particle. *Grapevine leafroll-associated virus-1* has a lower buoyant density than either GLRaV-2 or GLRaV-3, which have similar buoyant densities (Uyemoto *et al.*, 1997). This may be explained by the larger size of GLRaV-1. Grapevine leafroll-associated virus isolates belonging to the *ampelovirus* genus generally have a high molecular weight coat protein (CP) of between 32 and 43 kDa. *Grapevine leafroll-associated virus-2* has a CP M_r of 24 kDa (Boscia *et al.*, 1995; Choueiri *et al.*, 1996; Monis and Bestwick, 1997; Zimmermann *et al.*, 1990), which is consistent with the *closterovirus* genus (Martelli *et al.*, 2002).

The presence of high molecular weight dsRNA, from 8 x 10^6 M_r (Mossop *et al.*, 1985) to 10 x 10^6 M_r (Hu *et al.*, 1990a), is consistently associated with leafroll-infected vines (Choueiri *et al.*, 1996; Ling *et al.*, 1997; Hu *et al.*, 1990a; Mossop *et al.*, 1985; Rezaian and Krake, 1987). The large M_r dsRNA is most likely the replicative form of the genomic RNA. Lower M_r dsRNA molecules have been shown to be

subgenomic dsRNA components of the GLRaVs (Fazeli and Rezaian, 2000; Habili and Rezaian, 1995; Saldarelli, *et al.*, 1994). The isolation of dsRNA and the development of molecular genetic methods have facilitated the molecular characterisation of GLRaVs and the sequencing of many genes from the different *Closteroviridae* members.

Table 2.1 Physicochemical characteristics of GLRaVs.

Virion	Particle length	CP *Mr*	Genome size	Reference
GLRaV-1	2200 nm	39 kDa	ND	Zimmermann *et al.*, 1990
GLRaV-2	1800 nm	24 kDa	15.5 kb	Zimmermann *et al.*, 1990
GLRaV-3	1800 – 1900 nm	43 kDa	19 –20 kb	Zee *et al.*, 1987
GLRaV-4	1800 nm	36 kDa	ND	Zimmermann *et al.*, 1990
GLRaV-5	1400 – 1700 nm	36 kDa	ND	Boscia *et al.*, 1995
GLRaV-6	1800 nm	36 kDa	ND	Boscia *et al.*, 1995
GLRaV-7	1500 – 1700nm	37 kDa	19 –20 kb	Choueiri *et al.*, 1996
GLRaV-8	ND	37 kDa	ND	Monis and Bestwick, 1997
GLRaV-9	ND	ND	ND	Alkowni *et al.*, 2002

ND Not determined

The C*losteroviridae* have been divided into three genera, depending on insect transmissibility and genome organisation, namely the *closterovirus*, *crinivirus* and *ampelovirus* genera (Martelli *et al.*, 2002) (Fig. 2.1). Nine serologically distinct members of the *Closteroviridae* have been isolated from leafroll-infected grapevines (Alkowni *et al.*, 2002; Boscia *et al.*, 1995; Choueiri *et al.* 1996; Hu *et al.*, 1990a; Monis, 2000; Monis and Bestwick, 1997; Zimmermann *et al.*, 1990). These viruses differ in their phylogenetic and serological relationships and in genome organisation (Fazeli and Rezaian, 2000; Karasev, 2000; Ling *et al.*, 1998; Zhu *et al.*, 1998). Distant serological relationships between GLRaV-1 and GLRaV-3 (Seddas *et al.*, 2000) and GLRaV-4, GLRaV-5 and GLRaV-8 (Monis, 2000) have been observed in western blot assays performed with monoclonal antibodies. Sequence anlaysis indicates that GLRaV-9 shares 79% sequence similarity with GLRaV-5 (Alkowni *et al.*, 2002).

Nucleic acid-based homology determination between the coat protein (*CP*), RNA dependent RNA polymerase (*RdRp*), helicase (*Hel*) and heat shock protein 70 kDa homolog (*hsp70h*) genes have provided further data on the genetic diversity and relationships of these viruses (Fazeli and Rezaian, 2000; Karasev, 2000; Ling *et* al., 1998; Saldarelli *et al.*, 1998). Phylogenetic analysis based on the RdRp and Hel domains of the polymerase gene, *CP* and *dCP* genes confirmed the close relationship between GLRaV-1 and GLRaV-3 (Fazeli and Rezaian, 2000; Karasev, 2000). Phylogenetic analysis of the GLRaVs, shows that GLRaV-1, GLRaV-3, GLRaV-4 and GLRaV-5 are members of the *ampelovirus* genus (Karasev, 2000; Martelli *et al.*, 2002). From these phylogenetic trees it can be concluded that GLRaV-1 is most closely related to GLRaV-3 and that GLRaV-4 is most closely related to

GLRaV-5. These four viruses form two distinct but different clades within the *ampelovirus* genus (Fig. 2.1).

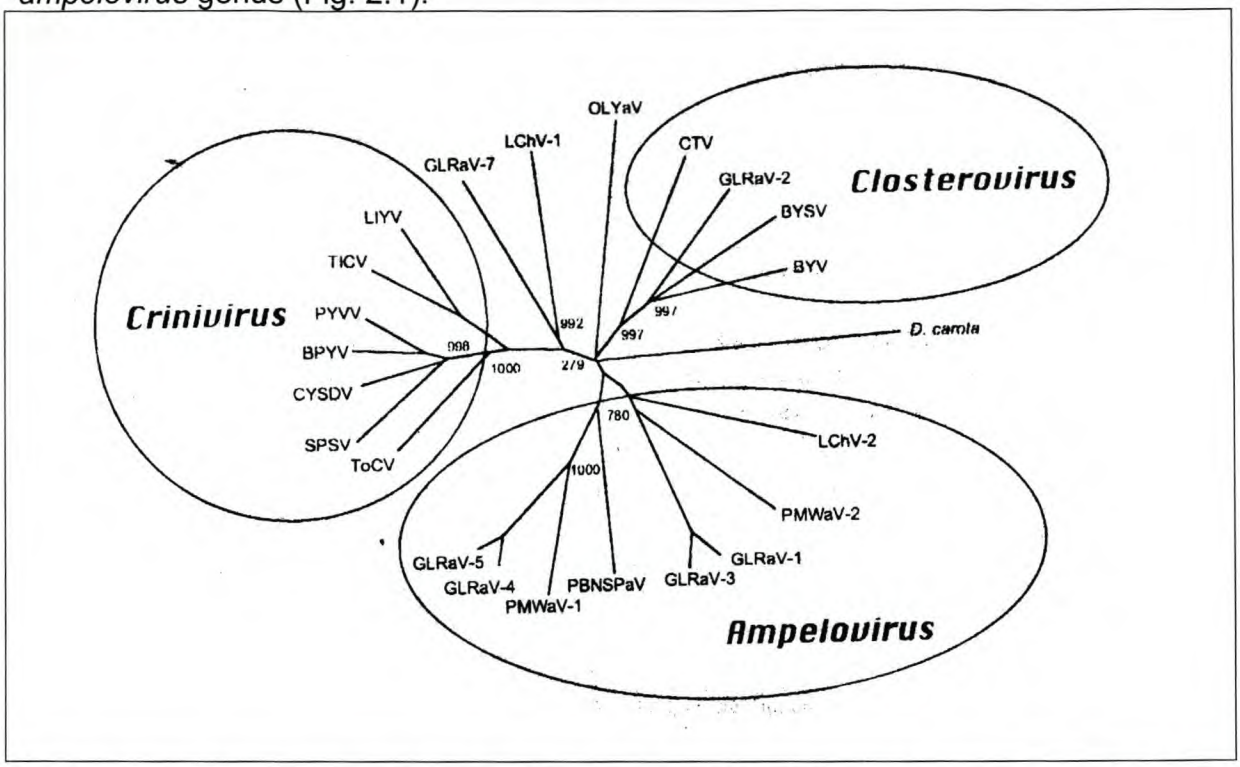

Fig. 2.1 Phylogenetic tree showing the relationship between the virus members and genera of the family *Closteroviridae* based on the sequence of the *hsp70h* gene. The neighbour joining tree was produced and bootstrapped using CLUSTAL W. The branch lengths are proportional to the sequence distances. GLRaV-7, LChV-1 and OLYaV are unassigned species in the family. *Daucus carota* has been used as an outgroup (Martelli *et al.,* 2002).

Grapevine leafroll-associated virus-2 has very high homology to a new grapevine infecting member of the *Closteroviridae*, grapevine rootstock stem lesion virus (GRSLV). These viruses are distinct from other members of the *closterovirus* genus, namely *beet yellows closterovirus* (BYV), *beet yellow stunt closterovirus* (BYSV) and *citrus tristeza closterovirus* (CTV).

Grapevine leafroll-associated virus-7 is an unassigned member of the *Closteroviridae.* Phylogenetic analysis of the *hsp70h* gene from GLRaV-7, however, indicates that it is most closely related to members of the *crinivirus* genus (Martelli *et al.*, 2002) (Fig 2.1).

A phylogenetic analysis study using the partial Hsp70h portion amplified with degenerate primers was performed by Saldarelli *et al.* (1998). These researchers identified a GLRaV-1 isolate which had greater homology to GLRaV-2 and a GLRaV-3 isolate with greater homology to GLRaV-1. Subsequent sequence analysis (Sefc *et al.,* 2000) and phylogenetic analysis (Karasev, 2000) of the GLRaV-1 and GLRaV-3 sequences provided by Saldarelli *et al.* (1998) have shown that these virus isolates should be reclassified as GLRaV-2 and GLRaV-1 respectively.

The insect transmissibility for most of these viruses is unknown at present. Only GLRaV-1 and GLRaV-3 have confirmed insect vectors. All of these viruses can be transmitted by grafting, but cannot be mechanically transmitted to grapevine or

herbaceous hosts. The exception is GLRaV-2, which can be mechanically transmitted to *Nicotiana benthamiana* (Goszczynski *et al.*, 1996). The latest taxonomy of the *Closteroviridae* indicates that GLRaV-2 is a species within the *closterovirus* genus. *Grapevine leafroll associated virus-1*, GLRaV-3 and GLRaV-5 are species, while GLRaV-4, GLRaV-6 and GLRaV-8 are tentative species within the *ampelovirus* genus. Grapevine leafroll associated virus-7 remains as an unassigned member of the *Closteroviridae* family. (Martelli *et al.*, 2002)

2.2.2 THE EFFECTS OF GRAPEVINE LEAFROLL DISEASE

Grapevine leafroll-associated viruses infect all members of the *Vitis* family and cause clearly defined symptoms on the leaves of red cultivars. Most white and rootstock cultivars, however, do not display clear symptoms, resulting in latent infections that can only be detected by sensitive detection techniques. The subsequent propagation of grapevines with latent infections results in the further spread of the disease (Pietersen, Pers. Comm. Agricultural Research Council - Roodeplaat).

2.2.2.1 Grapevine leafroll disease symptoms

Not all grapevine cultivars show leafroll symptoms to the same extent. Red cultivars tend to display the most characteristic symptoms, white cultivars have less pronounced symptoms, while many rootstock and French-American hybrid varieties do not exhibit any symptoms (Goheen, 1988; Kovacs *et al.*, 2001). Symptoms also vary with environmental conditions, virus isolates and viticultural practices (Alley *et al.*, 1963; Clingeleffer and Krake, 1992; Forsline *et al.*, 1996; Krake, 1993; Woodham *et al.*, 1973). Leafroll-infected vines are more sensitive to environmental stress than uninfected vines (Scheu, 1936). Distinct leafroll foliar symptoms usually appear in late summer or early autumn (Fig. 2.2). Foliar symptoms can be observed in early- to mid-summer if the vines are subjected to water or heat stress (Carstens, 2001; Golino, 1992). Symptoms first appear on the mature basal leaves of infected canes. These symptoms include the downward rolling of the leaf blade, increased leaf brittleness, interveinal reddening in red cultivars and interveinal chlorosis in white cultivars, with the veins remaining green (Goheen, 1988). Symptoms can also be expressed in the fruit, where the colour development of red fruit is delayed or reduced (Goheen, 1988). Grapevine leafroll-associated viruses can cause reduced root and vegetative growth, decreased sugar accumulation (Krake, 1993), reduced cluster weight and reduced cluster numbers, which reduce fruit yields. Wines made from leafroll-infected vines have been shown to have reduced alcohol, colour and tannin. These reductions were found to be inversely proportional to the severity of the disease symptoms (Reynolds *et al.*, 1997).

Krake (1993) characterised symptoms of leafroll from 19 different isolates on six indicator plants, namely Baco blanc 22A, Cabernet franc, Emperor, Mission, LN33 and Sultana. All leafroll isolates induced downward rolling of the leaf blade on indicator plants. Certain virus isolates induced symptoms in some indicator plants,

but not in others. Symptoms included the following: 1) interveinal colouration and / or necrosis, 2) interveinal reddening or chlorosis with distinct green veins, 3) reddening of the ends of the veins and leaf margins with interveinal reddening and 4) severe stunting of Baco blanc 22A plants. The severity of the interveinal reddening or chlorosis varied from mild to severe. At the end of autumn, some leaves became completely red. The variation of leafroll symptoms could have been due to either strain variation (Clingeleffer and Krake, 1992; Wolpert and Vilas, 1992; Woodham *et al.*, 1973), or to synergistic effects caused by mixed infections (Engelbrecht and Kasdorf, 1990a). There is, however, no clear correlation between symptom severity and virus isolate (Krake, 1993). Krake (1993) observed that the different isolates of grapevine leafroll cause similar disease symptoms in grapevine. It must be noted that particular GLRaVs were not identified from these different isolates. Phylogenetic analysis has shown that significant variation exists amongst the GLRaVs and these viruses belong to different genera within the *Closteroviridae*. This diversity of the GLRaVs supports the view that leafroll symptoms may be a general physiological response of the vine to phloem-limited pathogen infection, which interferes with nutrient movement in the vine as a result of phloem degeneration (Krake, 1993; Martelli, 1993). Furthermore, the phloem-limited virus diseases of leafroll, corky bark and rupestris stem pitting have overlapping symptoms, yet they are caused by different viruses (Goheen, 1988). It would be interesting to determine whether symptom severity correlates with the severity of phloem degeneration caused by leafroll-associated *Closteroviridae*. To date, this aspect has not been examined.

Fig. 2.2 Typical leafroll symptoms showing interveinal reddening and clear green veins expressed on a red wine cultivar in late summer.

Grapevine leafroll disease does not directly result in vine death. It is, however, indirectly responsible for the reduction in the productive lifespan of grapevines, which

can in turn result in more frequent re-establishment of vineyards, hence increased demand on labour and additional costs for grape growers.

2.2.2.2 Anatomic and cytopathological effects of grapevine leafroll disease

Grapevine leafroll causes phloem degeneration, the collapse of phloem cells and sieve cell necrosis (Hoefert and Gifford, 1967a). Phloem degeneration is the primary anatomical effect of leafroll infection and occurs in the vascular bundles of leaves, stems and fruit pedicels. Although a natural plant process, phloem degeneration occurs earlier and is more pronounced in leafroll-infected vines (Hoefert and Gifford, 1967a). The extent of phloem degeneration has been shown to be less severe in older internodes and higher in older leaves. Primary phloem appears to be more susceptible to leafroll infection than secondary phloem (Hoefert and Gifford, 1967a).

Phloem degeneration can be observed before the onset of symptoms in the leaves. Secondary symptoms include hypertrophy and hyperplasia of phloem parenchyma, production of gum, tyloses and trabeculae in the xylem elements, and the accumulation of starch. The gum-like deposit is a product of cell degeneration. The formation of tylose and trabeculae is normal, but appears earlier in virus-infected plants. The early accumulation of tylose indicates that virus infection accentuates phloem degeneration (Hoefert and Gifford, 1967b). Starch accumulation in infected leaves occurs in the spongy parenchyma before the onset of symptoms (Hoefert and Gifford, 1967b). It has not been established whether starch accumulation occurs before or after phloem degeneration. However, phloem degeneration would inhibit translocation and could therefore result in starch accumulation and brittleness of the leaves. Early tylose formation can prevent the translocation of ions into leaves. These anatomical changes can be partially responsible for the physiological effects and the outward morphological symptoms displayed by leafroll-infected vines (Honey, Unpublished, Department of Viticulture and Oenology, University of Stellenbosch).

Leafroll-infected grapevines have been reported to contain virus-like particles and cytopathic structures typically seen in plants infected with members of the *Closteroviridae* (Francki *et al.*, 1985). Elongated virus particles typical of the *Closteroviridae* were found in the cytoplasm of the sieve elements, companion cells and phloem parenchyma cells of leafroll-infected grapevines. Virus particles have also been found in leaves that did not display symptoms, although virus particles have never been seen in virus-free grapevines with symptomless leaves (Kim *et al.*, 1989; Zee *et al.,* 1987). In older symptomatic leaves, the parenchyma cytoplasm was entirely replaced by virus particles (Zee *et al.,* 1987). Bundles of filamentous particles were found in the cytoplasm of differentiating sieve tubes and companion cells (Castellano *et al.*, 1983; 2000). Virus particles were often packed parallel along their long axis, but were also found to be arranged in a disorderly manner (Zee *et al.,* 1987).

The cytopathic effects are phloem limited and are found in the sieve tubes, companion cells and phloem parenchyma cells, and consist of vesicles of 50 to 100

nm in diameter (Kim *et al.*, 1989). These virus-induced vesicles are the site of viral replication and are a reliable feature of virus infection (Martelli and Bar-Joseph, 1991). These vesicles are formed in modified mitochondria (Kim *et al.*, 1989). The phloem parenchyma of young leaves contained some vesicles without the virus-like particles or electron-dense granular material. However, most vesicles in the young leaves contained electron-dense fibrils (Kim *et al.*, 1989). Similar ultrastructural modifications were observed in *Nicotiana benthamiana* phloem tissue infected with GLRaV-2 (Castellano *et al.*, 2000).

2.2.2.3 Effect of grapevine leafroll disease on grape and wine quality

Leafroll disease can affect the physiological growth of grapevines. Leafroll-infected vines show delayed bud break and flowering (Over de Linden and Chamberlain, 1970; Woodham *et al.*, 1983), and reduced vigour (Lider *et al.*, 1975; Over de Linden and Chamberlain, 1970) when compared to uninfected vines. Virus-free vines yield a wider, greener canopy with better photosynthetic potential than virus-infected vines. The reduction of photosynthetic activity in leaves occurs before the appearance of symptoms (Guidoni *et al.*, 1997; 2000). The symptoms of leafroll, interveinal reddening and chlorosis and the downward rolling of leaf blades, are considered a consequence of, and not the cause of reduced photosynthetic potential, leaf chlorophyll content and vegetative vigour (Guidono *et al.*, 2000). Leafroll-infected vines also have smaller roots (Over de Linden and Chamberlain, 1970), which retard nutrient uptake and reduce the auxin and cytokinin production required for growth regulation. Smaller roots also upset the balance between root and vegetative growth (Archer, Pers. comm. Department of Viticulture and Oenology, University of Stellenbosch) and may result in smaller vines as observed in reduced vegetative growth.

The effect of leafroll infection on grape and wine quality varies with cultivar (Over de Linden and Chamberlain, 1970), growth season (Cabaleiro *et al.*, 1999), symptom severity (Krake, 1993) and virus isolate (Guidoni *et al.*, 1997; 2000). Grapevine leafroll infection reduces grape yields in Mission and Baco blanc 22A (Over de Linden and Chamberlain, 1970), Burger (Kliewer and Lider, 1976; Lider *et al.*, 1975) and Sultana cultivars (Woodham *et al.*, 1984). Sultana vines expressing severe symptoms had significantly lower fruit yields than vines expressing mild symptoms (Woodham *et al.*, 1983; 1984).

Leafroll infection results in a decrease in fruit sugar content (Cabaleiro *et al.*, 1999; Clingeleffer and Krake, 1992; Guidoni *et al.*, 1997; Hale and Woodham, 1979; Kliewer and Lider, 1976; Krake, 1993; Kovacs *et al.*, 2001; Lider *et al.,* 1975; Over de Linden and Chamberlain, 1970; Wolpert and Vilas, 1992). This reduced sugar content is usually associated with higher acidity levels, which are an indication of delayed fruit maturation (Guidoni *et al.*, 1997; Kliewer and Lider, 1976; Kovacs *et al.*, 2001; Lider *et al.,* 1975; Over de Linden and Chamberlain, 1970). Fruit maturation may be delayed by up to 20 days and is evident three months prior to cropping

(Kliewer and Lider, 1976; Lider *et al.*, 1975). This decrease in sugar content and increase in acidity levels became less pronounced as the grapes approached harvest (Over de Linden and Chamberlain, 1970). Delayed fruit maturity and decreased sugar accumulation have been found to be more pronounced in adverse weather conditions (Cabaleiro *et al.*, 1999). Delayed maturity may, however, be overcome by crop thinning (Kliewer and Lider, 1976; Lider *et al.*, 1975).

Leafroll infection can reduce berry pigmentation (Krake, 1993; Over de Linden and Chamberlain, 1970) and interfere with phenol and anthocyanin metabolism. This results in decreased anthocyanin levels in berry skins and increased anthocyanin levels in foliar tissue (Guidoni *et al.*, 1997; 2000), resulting in paler red wines (Over de Linden and Chamberlain, 1970) and lower red wine quality (Guidoni *et al.*, 1997).

Grapevine leafroll-associated virus-3 mixed with *grapevine A vitivirus* (GVA) was found to have a pronounced effect on fruit quality, whilst GLRaV-1 mixed with GVA had a significant effect on fruit quantity (Guidoni *et al.*, 1997; 2000). This may explain the variation seen with respect to the effect of leafroll infection on fruit quantity and quality noted by other researches.

There are reports that leafroll disease may be used as a devigorating agent in the field (Golino, 1992). This practice is detrimental to the growth of vines, due to the degeneration of phloem tissue (Hoefert and Gifford, 1967a; Goheen and Cook, 1959), and should be avoided and strongly discouraged. A better method of reducing vine vigour would be to use accepted viticultural practices, such as crop thinning and canopy management.

Although most studies have been conducted on young vines, the long-term effect of reduced photosynthesis and phloem degeneration on infected vines is bound to reduce vine vigour, crop yield and the lifespan of the vine (Cabaleiro *et al.*, 1999). This aspect of the effect of leafroll infection on vines has not been studied and needs to be investigated further.

2.2.3 VIRUS DISTRIBUTION IN LEAFROLL-INFECTED GRAPEVINES

Grapevine leafroll-associated virus-3 can be maintained in cane phloem tissue for up to six months in dormant vines and can be detected in flowers and roots 15 days after bud break, but cannot be detected in leaves at the start of vegetative growth (Teliz *et al.*, 1987). This initial movement from the cane phloem to the flowers can be explained by normal source-to-sink virus movement patterns. According to Teliz *et al.* (1987), GLRaV-3 was consistently detected in the basal leaves of vines 38 days after bud break, and in all basal and apical leaves 85 days after bud break through to harvest. The virus titre in younger apical leaves was consistently lower than the virus titre in older basal leaves. Throughout the growth season, GLRaV-3 was detected in roots, bark, leaves, fruit peduncles and fruit. Virus titres decreased in fruit, but increased in bark and leaves over the growth season (Teliz *et al.*, 1987). In a further study, GLRaV-3 was found to be unevenly distributed and virus titres fluctuated throughout the growth season (Monis and Bestwick, 1996). This uneven distribution

may be due to inefficient movement or inefficient replication of the virus in the phloem tissue (Monis and Bestwick, 1996).

Virus movement in grapevines appears to be divided into three periods: 1) bud break to inflorescence swelling, when the virus is restricted to flower clusters; 2) fully developed inflorescences to developing berries, when the virus was detected in basal leaves but not in apical leaves; 3) berry touch stage to harvest, when the virus was detected in basal and apical leaves (Teliz *et al.*, 1987). An understanding of the distribution of GLRaVs within the grapevine is important for the accurate detection of the viruses in grapevines.

2.2.4 INCIDENCE AND SPREAD OF GRAPEVINE LEAFROLL DISEASE

Grapevine leafroll disease is found in all areas in which grapes are grown (Goheen, 1988) and is widespread in most of these regions (Table 2.2). The level of leafroll infection varies within a vineyard block, between vineyard blocks, between viticultural areas and between countries. Of the nine GLRaVs, GLRaV-1 and GLRaV-3 are most frequently detected in leafroll-infected grapevines (Boscia *et al.*, 1995; Credi and Giunchedi, 1996; Zimmermann *et al.*, 1990). *Grapevine leafroll-associated virus-2* is less widespread than GLRaV-1 and GLRaV-3 and may be associated with rootstock-scion incompatibility disorder (Greif *et al.*, 1995) and leafroll disease (Boscia *et al.*, 1995). The distribution and incidence of the different GLRaVs varies with geographical region. Grapevine leafroll-associated viruses that are prevalent in some areas may be absent from others. Some GLRaVs may have a distinct association with a particular cultivar (Boscia *et al.*, 2000).

A survey of leafroll disease in South Africa showed that 68.4% of wine and rootstock cultivars were infected with GLRaVs (Nel and Engelbrecht, 1972). The high incidence of leafroll infection was due to latently infected rootstocks. Self-rooted scions showed a reduced level of leafroll infection when compared to scions that had been grafted onto rootstocks. A survey, currently being undertaken in the South African industry on the spread of leafroll in certified vines, shows that GLRaV-3 is present in vineyard blocks supplying propagating material (Pietersen, 2002). The percentage of GLRaV-3 infected vines in mother blocks varied between 0 and 29.3%, with an average infection rate of 1.58%. This average, though low, is too high for blocks from which "certified" material is released. The rate of increase of leafroll infection can be up to 1.22% per annum within these blocks (Pietersen, 2002). *Grapevine leafroll-associated virus-3* was found in mixed infections with GVA in 25% of cases and with other unidentified elongated particles in 10% of cases. Few vineyard blocks showed clear, random distribution patterns of GLRaV-3, which indicates that the infection was initiated from infected planting material. Most vineyard blocks, however, showed distribution patterns indicative of secondary spread of leafroll by vectors (Pietersen, 2002). In South Africa, plant improvement schemes do not guarantee that leafroll-free plant material will reach producers, because mother blocks are not declared 100% virus free (Van der Westhuizen, 2002). However, the

Table 2.2 Distribution of grapevine leafroll disease around the world (Taken from the Crop Protection Compendium 2002, http://cabicompendium.org/cpc/)

Region		Distribution	Reference
Asia			
Armenia		widespread	Digiaro *et al.*, 2000
China		present	
	Henan	widespread	Li *et al.*, 1989
	Liaoning	widespread	Li *et al.*, 1989
	Taiwan	widespread	Tzeng *et al.*, 1994
Gaza		widespread	Alkowni *et al.*, 1998
Israel		widespread	Tanne and Nitzany, 1973
Japan		widespread	Tanaka, 1976; Namba *et al.*, 1979
Jordan		widespread	Al-Tamimi *et al.*, 1998
Republic of Korea		widespread	Kim *et al.*, 2000
Lebanon		widespread	Haidar *et al.*, 1996
Turkey		widespread	Azeri, 1990; Ozaslan *et al.*, 1993
Yemen		restricted	Martelli *et al.*, 1994
Europe			
Austria		widespread	Flak and Gangl, 1994
Bulgaria		widespread	Abracheva, 1977
Croatia		widespread	Saric and Korosec-Koruza, 1991
Cyprus		widespread	Ioannou, 1993
Czechoslovakia		widespread	Blattny *et al.*, 1960
Former Yugoslavia		widespread	Dimitrijevic, 1970
France		widespread	Vuittenez, 1958; Zimmermann *et al.*, 1988
Germany		widespread	Scheu, 1936; Kassemeyer, 1991
Greece		widespread	Katis *et al.*, 1991
Hungary		widespread	Lehoczky *et al.*, 1969
Italy		widespread	Belli and Cesati, 1967; Faoro *et al.*, 1981
Malta		widespread	Martelli *et al.*, 1992
Moldova		widespread	Bondarchuk *et al.*, 1991
Portugal		widespread	Boehm and Martins, 1991
	Azores	widespread	Lopes *et al.*, 1997
Romania		widespread	Pop *et al.*, 1993
Slovenia		widespread	Saric and Korosec-Koruza, 1991
Spain		widespread	Cabaleiro and Segura, 1997a
Switzerland		widespread	Bovey, 1968; Gugerli *et al.*, 1984
Ukraine		widespread	Milkus *et al.*, 1991
Africa			
Algeria		widespread	Digiaro *et al.*, 2000
Morocco		widespread	Digiaro *et al.*, 2000
South Africa		widespread	Nel and Engelbrecht, 1972
Tunisia		widespread	Agran *et al.*, 1990; Mahfoudhi *et al.*, 1998
North America			
Canada		present	
	British Columbia	widespread	MacKenzie *et al.*, 1996
	Nova Scotia	restricted	MacKenzie *et al.*, 1996
	Ontario	widespread	MacKenzie *et al.*, 1996
	Quebec	restricted	MacKenzie *et al.*, 1996
Mexico		widespread	Teliz *et al.*, 1989
USA		present	
	California	widespread	Hewitt, 1954
	Missouri	widespread	Milkus and Goodman, 1999
	New York	widespread	Wilcox *et al.*, 1998
South America			
Brazil		widespread	Kuhn, 1989; Scagliusi, 1995
	Rio Grande do Sul	widespread	Kuhn, 1989
Chile		widespread	Auger *et al.*, 1989
Oceania			
Australia		present	
	New South Wales	widespread	Fraser, 1958
	South Australia	widespread	Habili *et al.*, 1996
	Victoria	widespread	Habili *et al.*, 1996
New Zealand		widespread	Chamberlain, 1967; Mossop *et al.*, 1985

improved regulations regarding the sale of certified planting material and improved detection regimes may help to provide grape growers with the best possible material under the circumstances. Similar surveys in other parts of the world have been conducted. In Australia and New Zealand, the incidence of leafroll disease is much lower than in South Africa (Habili *et al.*, 1996; 2002).

The spread of leafroll disease is due mainly to the planting of virus-infected material or the top grafting of undesirable cultivars. The spread of leafroll disease in vineyards has been noted throughout the world, but is significant in vineyards in Australia (Habili *et al.*, 1995; Habili and Nutter, 1997), Italy (Belli *et al.*, 1994), New Zealand (Jordan, 1993; Bonfiglioli *et al.*, 2002a; 2002b), South Africa (Engelbrecht and Kasdorf, 1990b) and Spain (Cabaleiro and Segura, 1997a). The spread of leafroll disease in the field has only been confirmed for GLRaV-1 (Bonfiglioli *et al.*, 2002b) and GLRaV-3 (Bonfiglioli *et al.*, 2002a; Cabaleiro and Segura, 1997a; Engelbrecht and Kasdorf, 1990b; Habili *et al.*, 1995; Habili and Nutter, 1997; Jordan, 1993). This is due to the known vector transmissibility of these two viruses.

The spread of leafroll disease can result in disease incidence increasing from 0 to 21.3% over a three-year period after planting (Cabaleiro and Segura, 1997b). Whether this increase was due to late symptom expression, increased virus titres or leafroll spread is not known.

The rate of spread in New Zealand is particularly high, with an overall annual increase of between 10 to 12% (Bonfiglioli *et al.*, 2002a). This spread is non-random and occurs mainly along the rows, but can occur across rows (Jordan, 1993). Jordan (1993) also found that mealybugs are present in high numbers in New Zealand vineyards. *Grapevine leafroll-associated virus-1* appears to spread downwind in New Zealand vineyards (Bonfiglioli *et al.*, 2002b).

If infected vines are removed when infection is first detected, virus levels remain relatively constant. However, if infected material is not removed, the level of infection can increase dramatically (Kingston, 2002). If the vines are not removed, the spread of leafroll disease can result in a doubling of virus infection in successive years (Jordan, 1993). The rate of leafroll spread is dependent on the level of initial infection and / or the level of vector that is present. The distribution of leafroll disease can be random, due to the random planting of diseased material (Bonfiglioli *et al.*, 2002a; Jordan, 1993), or can form a disease gradient due to the transmission of the virus by insect vectors (Pietersen, 2002).

2.2.5 VECTOR TRANSMISSION OF GRAPEVINE LEAFROLL DISEASE

Grapevine leafroll-associated virus-1 and GLRaV-3 are transmitted in the field by a variety of mealybug and scale insects (Krake *et al.,* 1999) (Table 2.3). The ability of mealybugs to transmit GLRaVs is due more to mealybug-virus interaction than to mealybug-grapevine interaction (Petersen and Charles, 1997). These insects may transmit one or both of GLRaV-1 and GLRaV-3 in a single event (Sforza *et* al., 2000). Insect transmission of the other seven GLRaVs has not been confirmed. *Grapevine*

leafroll-associated virus-2 has been shown to be acquired by *Pseudococcus ficus*, but cannot be transmitted by this mealybug (Engelbrecht and Kasdorf, 1990b). Phylogenic relationships generated for the *Hel*, *RdRp* and *hsp70h* genes suggest that there could be a strong correlation between the phylogenies of these proteins and vector transmission. Based on these phylogenic relationships, it could be possible to predict the mode of transmission of the GLRaVs for which insect vectors are unknown (Karasev 2000). *Grapevine leafroll-associated virus-2* groups with the aphid-transmissible *closterovirus* genus, GLRaV-4 and GLRaV-5 group with the mealybug-transmissible *ampelovirus* genus and GLRaV-7 groups with the whitefly-transmissible *crinivirus* genus. This prediction still needs to be proven and may explain why mealybug vectors for all GLRaVs have not been identified. This hypothesis is not based on the insect transmissibility factor, which still needs to be identified.

Mealybugs are able to transmit GLRaVs from vines with low virus titres throughout the growth season (Kingston, 2002; Petersen and Charles, 1997). Field spread can be obtained when insect vectors are found in low population numbers. Five to 10 *Pseudococcus calceolariae* can transmit GLRaV-3 to a new plant (Petersen and Charles, 1997). This indicates that virus transmission is efficient. Higher virus transmission is achieved with higher numbers of insect vectors (Cabaleiro and Segura, 1997b) or from plants with higher virus titres (Kingston, 2002).

Table 2.3 Insect vectors of GLRaV-1 and GLRaV-3

Virus	Insect Vector	Reference
GLRaV-1	Scale - *Neopulvinaria innumerabilis*	Fortusini *et al.*, 1997
	Scale - *Parthenolecanium corni* Bouché	Fortusini *et al.*, 1997
		Sforza *et al.*, 2000
	Mealybug – *Heliococcus bohemicus* Sulc	Sforza *et al.*, 2000
	Mealybug – *Phenacoccus aceris* Signoret	Sforza *et al.*, 2000
GLRaV-3	Mealybug - *Pseudococcus longispinus*	Tanne *et al.*, 1989
		Petersen and Charles, 1997
	Mealybug – *Plannococcus ficus*	Engelbrecht and Kasdorf, 1990a
	Scale – *Pulvinaria vitis*	Belli *et al.*, 1994
	Mealybug – *Pseudococcus viburni*	Golino *et al.*, 1995
	Mealybug – *Pseudococcus calceolariae*	Petersen and Charles, 1997
	Mealybug – *Planococcus citri*	Cabaleiro and Segura, 1997a, b
	Mealybug – *Heliococcus bohemicus*	Sforza *et al.*, 2000
	Mealybug – *Pseudococcus maritimus*	Golino *et al.*, 2000

Grapevine leafroll-associated virus-3 is transmitted most efficiently by the first instar stage of mealybugs and scale insects, but can be transmitted by other instar stages (Kingston, 2002; Petersen and Charles, 1997). The first instar stage of *Planococcus longispinus* can transmit GLRaV-3 with an efficiency of 55% (Kingston, 2002) and the probability of transmission from viruliferous *Pl. citri* is similar to the reported values of other vector / plant systems (Berger and Ferriss, 1988). The

acquisition feeding time for *Pl. citri* is 72 h, and although the mealybug can retain the virus for 24 hours, it loses the ability to transmit the virus to new plants after one hour (Cabaleiro and Segura, 1997b). This indicates that GLRaV-3 is transmitted semi-persistently by mealybugs. The sedentary nature of mealybugs results in the slow spread of GLRaV-1 and GLRaV-3. First instars, however, commonly move by wind dispersion (Habili *et al.*, 1995; Milne *et al.*, 1984). This can account for the inconsistencies observed in virus distribution patterns (Carstens, Pers. comm. Agricultural Research Council – Infruitec-Nietvoorbij). Mealybugs generally complete two generations per year. This can result in the wind dispersal of the first instar during two periods in the year and the subsequent spread of GLRaV-1 and GLRaV-3 over a large distance. Knowledge of the mealybug life cycle and of the transmission ability of the different instar stages can be used to control the spread of leafroll through the appropriate control and management of the insect vector.

2.2.6 VIRUS DETECTION IN LEAFROLL-INFECTED GRAPEVINES

Disease detection is important for the identification and management of grapevine leafroll disease, the production of planting material, the release of virus-free cultivars and the quarantine of diseased material. The successful management of grapevine leafroll is dependent on accurate and early detection of the disease or disease-causing agents. Early detection is important to avoid the spread of leafroll to new and/or existing vineyards (Martin *et al.*, 2000). Accurate detection of grapevine leafroll disease is also important to determine the rate of spread of the disease, which is essential for the determination of the effectiveness of management practices that are used to control grapevine leafroll disease.

Numerous techniques can be used to detect GLRaVs. These include symptomology, biological indexing, electron microscopy, enzyme-linked immunosorbant assays (ELISA) and polymerase chain reaction-based and hybridisation-based molecular techniques. However, the effectiveness of disease detection by all of these techniques is dependent on the sampling procedure.

A major problem in the detection of the GLRaVs is the variable distribution of the viruses in grapevine tissue (Monis and Bestwick, 1996; Rowhani *et al.,* 1997). The highest level of virus is detected in the phloem tissue of canes, followed by the petioles and midribs of older symptomatic basal leaves. Higher virus titres are found in basal samples, whilst lower virus titres are found in apical samples of the vine (Hu *et al.*, 1991; Ling *et al.*, 2001; Monis and Bestwick, 1996; Teliz *et al.*, 1987). Virus titres also vary with the growth season. Initial virus titres are low and the titres peak in early summer at the maximum berry size. Therefore, in order to accurately detect GLRaVs, a range of tissue samples or samples from the correct part of the vine, taken at the appropriate time of year, need to be collected for testing (Gugerli, 2000). Bark scrapings from basal portions of the canes of symptomatic and non-symptomatic vines are the most reliable source of tissue for detection of GLRaVs (Habili and Randles, 2002; Ling *et al.*, 2001).

Symptomology is limited to the time of year, cultivar, viticultural practices and virus titre. Symptomology is the easiest and cheapest way of monitoring fields that have a known virus history. Symptomology is not reliable for the accurate detection of leafroll disease and should not be used for this purpose. Biological indexing is reliable, but is time consuming. A major problem with biological indexing is the variable virus distribution within the grapevine. This may result in virus-free buds being grafted onto indicator hosts and the subsequent failure to detect the virus in the tested indicator host. An advantage of biological indexing over the laboratory tests arises from the broad-spectrum detection of leafroll disease as opposed to specific virus isolates.

Laboratory tests such as ELISA and PCR-based techniques are sensitive and can give results in one to two days. These techniques also allow large numbers of samples to be tested, allowing for increased information on the distribution, incidence and movement of GLRaVs within vineyards. The ease of use and mechanisation of ELISA has made this technique very suitable for wide-scale tests. ELISA is therefore the most common technique used to date, particularly for the detection of GLRaV-1 and GLRaV-3, for which reliable antibodies are available. Electron microscopy, reverse transcription-polymerase chain reaction (RT-PCR) and nucleic acid hybridisation are useful for verifying questionable samples that do not give definitive results in ELISA (Ling *et al.*, 2001). A study comparing biological indexing and ELISA showed perfect correlation between symptom development on indicator plants and ELISA results for GLRaV-1, GLRaV-2 and GLRaV-4. However, 49 plants tested positive for GLRaV-3 with biological indexing and 57 plants tested positive with ELISA. The eight samples that gave variable results were retested by IC-RT-PCR and all of these plants tested positive for GLRaV-3 (Rowhani *et al.*, 1997). A study performed by Ling *et al.* (2001) showed that RT-PCR could be used to validate the results obtained for GLRaV-3-infected plants by biological indexing and ELISA. From these results, it is evident that RT-PCR is as sensitive as ELISA, but more sensitive than biological indexing.

The use of reliable and sensitive detection techniques such as ELISA and RT-PCR can result in the early detection of leafroll disease before the onset of symptom expression (Habili *et al.*, 1997). This can result in improved plant quarantine, leafroll management and plant production and a subsequent reduction in the incidence of leafroll disease (Martin *et al.,* 2000). Furthermore, the use of more than one sensitive method is advisable to eliminate GLRaV from grapevine and to assure producers of the best available material for planting that does not contain a latent infection.

ELISA is the preferred detection method for the diagnosis of plant virus disease because of its simplicity and effectiveness. However, RT-PCR is gaining popularity due to its increased sensitivity and the reliability of the results that are produced, the increasing number of available sequences for which primers can be made and the ability to design degenerate primers for broad-spectrum detection. The PCR-based techniques have also shown their usefulness in the identification of a new GLRaV

isolate (GLRaV-9) that could not be identified and detected by standard techniques (Alkowni *et al.*, 2002).

It is recommended that detection schemes move from ELISA to RT-PCR techniques for reliable virus detection in plant propagation and virus certification schemes, particularly because RT-PCR can reliably detect GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 and GLRaV-5. ELISA, on the other hand, can only reliably detect GLRaV-1 and GLRaV-3 (Habili *et al.*, 2002; Martin *et al.*, 2000; Weber, 2002). Furthermore, the RT-PCR detection technique compares favourably with ELISA with respect to cost, speed, accuracy and the skill level of operators.

The association of nine different GLRaV isolates with leafroll disease creates problems with disease detection and the generation of virus-free propagating material. Tests that are able to identify specific virus isolates are of limited practical value in sanitation and quarantine programmes. Vines that test negative for GLRaV-3, for instance, may still carry other GLRaVs, *vitiviruses* or *nepoviruses*. Therefore, broad-spectrum detection of diseases, rather than of the virus causing the disease, is still of practical value to the grapevine propagation industry. Once all known virus pathogens of grapevine have been identified, RT-PCR may replace biological indexing as the detection method of choice for broad-spectrum detection. This is because numerous RT-PCR tests can be performed from a single sample preparation and a single set of degenerate primers can be used to detect a variety of members of the *Closteroviridae*.

2.3 CONTROL OF GRAPEVINE LEAFROLL DISEASE

Viruses are economically important because they can cause systemic disease and can persist in the vegetative parts of the plant for as long as the plant remains alive. Once plants are infected, they remain infected indefinitely. This is evident in the perennial grapevine, which, although pruned each year, maintains virus infection. The status of GLRaV infection in South African vineyards does not appear to have improved significantly over the past 20 to 30 years. This is despite the supply of "virus-free" material and the introduction of grapevine certification schemes. The increased sensitivity and reliability of detection techniques allow surveys to be conducted that can highlight the sanitary status of vines and the spread of disease within vineyard blocks and grape-growing regions. Knowledge of the sanitary status of vines and the vector transmission of GLRaVs can be used to develop strategies that can control the spread of leafroll disease and help eliminate the disease from grape-growing areas. The supply of "virus-free" material to grape growers is not sufficient to control the problem of leafroll disease because of the occurrence of re-infection. "Virus-free" grapevines that are planted in an area that has a high incidence of leafroll disease are bound to become re-infected within a short period, therefore, other measures are required to control grapevine leafroll. These measures include the control of vectors that may transmit the viruses, elimination of virus sources,

general sanitation measures, grower education and the use of new technologies, such as genetic manipulation of grapevines, to improve tolerance to and control of GLRaVs. Unfortunately, it is virtually impossible to completely eradicate the source of grapevine leafroll and the control of leafroll disease will therefore be an ongoing venture.

2.3.1 MANAGEMENT STRATEGIES

Plant viruses are hard to control and infections are difficult to prevent. As with all plants and plant viruses, grapevines infected with GLRaVs cannot be cured. Currently, the best form of leafroll disease control is prevention of infection. Most preventative strategies are based on plant sanitation and vector control. However, this is not always sufficient, as seen in South African vineyards, where "re-infection" is a major problem. Therefore, management strategies are needed to limit the spread of GLRaVs and to limit the effect of leafroll disease on grapevines.

Preventative, control and management strategies need to be tailored to individual vineyard blocks, depending on the severity of the disease, the incidence of infection, the presence of vectors and the age of the vineyard. Management strategies may therefore, play a significant role in improving the quality of fruit from leafroll-infected vines and the quality of the subsequent wine.

The best quality fruit can be obtained from uninfected, healthy grapevines. Strategies that prevent infection are of particular importance when new vineyards are established and in young established vineyards. Preventative strategies need to be applied before the vines are planted and need to be maintained throughout the lifespan of the vineyard. Soil preparation and the choice of planting material are important elements in preventing virus infection. Infected roots may give rise to infected shoots, which provide a virus source for mealybug transmission. Grapevine roots can also be a source of the mealybug vector (Walton, 2001). Therefore, grapevine roots should be killed with an application of an appropriate herbicide and these roots should be completely removed before new vine material is planted. This can reduce the sources of both the virus and the mealybug vector. Weed control should be practiced, as certain herbaceous weeds may provide an alternative host for mealybugs (Walton, 2001).

New planting material should be certified as free of viruses and should be obtained from respected, certified nurseries that use the best and most sensitive detection methods to detect virus infection. It is essential that the mother blocks, from which these nurseries obtain their material, maintain a virus-free status. Grapevines should not be top-grafted to change the top cultivar. This practice should be discouraged. However, if top-grafting is necessary, all rootstocks and scion material should be tested for its virus status using the best detection techniques.

Virus-free vines can be produced by conventional heat therapy, meristem culture or a combination of these two techniques. If performed carefully, these techniques can eliminate viruses from plant material. However, they are not efficient for the

large-scale elimination of viruses from vines required for propagation. The production of "virus-free" vines is insufficient to control leafroll disease, because insect vectors can transmit GLRaVs and therefore re-infect these "virus-free" vines. This is particularly true if new "virus-free" vines are planted in close proximity to an established vineyard that is heavily infected or in a region that has a high incidence of leafroll disease.

In established young vineyards, good strategies should be implemented for the control of mealybug vectors. These include monitoring, chemical control of mealybugs at the appropriate time to have the most significant effect on mealybug populations (http://www.ipw.co.za/), and removal of herbaceous weeds, which may act as alternative mealybug hosts (Walton, 2001). Monitoring of vineyards for mealybug should be encouraged to reduce the amount of insecticide sprayed on the vines. Insecticides should only be sprayed when the mealybug population increases beyond the economic threshold (Kingston, 2002). It should be noted that it is impractical to eradicate mealybugs from vineyards, but that mealybugs should rather be maintained at levels below their economic threshold. Mealybugs should be killed with an effective insecticide at bud break in spring, when young instars are most susceptible to insecticides and come to feed at the base of buds. This needs to be repeated in mid-summer, when the second generation of mealybugs hatch and move to feed on shoots and grape clusters. In areas where a high level of mealybug infestation is noted, dormant vines can also be sprayed. Monitoring of mealybug populations is also important because mealybugs can be sprayed when their numbers rise above their economic threshold. The symbiotic relationship between mealybugs and ants requires that ant populations be effectively controlled for the effective control of mealybugs and leafroll disease (Walton, 2001). Once mealybug levels are below the economic threshold, insecticide sprays can be reduced and biological control, through predatory insects and parasites, can be utilised as an environmentally safe practice (Walton, 2001). Although mealybugs and scale insects are the only reported vectors of leafroll, it is possible that whitefly and aphids may also vector certain GLRaVs, which means that control strategies for whitefly and aphids should also be kept in mind for the effective control of leafroll disease.

Control and management strategies need to be employed to keep the level of virus infection to a minimum. The most important of these is the prompt removal of infected material. If infected vines are removed from a field, infection levels can be maintained at levels of 1 to 3%. However, if infected vines are not removed, virus levels in an established vineyard may increase from 3 to 19% over a six-year period (Kingston, 2002).

Although virus infection is detrimental to the quality of the fruit and subsequently to the quality of the wine, it is impractical and uneconomical to remove all infected vines from a heavily infected, young established vineyard. Therefore, viticultural management practices need to be applied to ensure the best quality of fruit from virus-infected vineyards. Viticultural practices that favour fruit ripening may minimise

the effect of leafroll on fruit quality. These practices include crop control (Kliewer and Lider, 1976; Lider *et al.*, 1975), pruning system (Woodham *et al.*, 1983), canopy management, irrigation and fertilisation, stress reduction, leaf removal and vegetative growth promotion. The effect of these viticultural practices on virus levels is unknown, although their effect on fruit quality is well established (Hunter and Visser, 1990, Smart *et al.*, 1990; Vasconcelos and Castagnoli, 2001). If virus-infected vines are to be left in vineyard blocks, strict attention needs to be paid to general sanitation to prevent the spread of the disease within the block. Other viticultural practices, such as general sanitation, movement from lightly infected to heavily infected vineyard blocks and the breaking of wind dispersion patterns, may be used to limit the spread of leafroll disease between vineyard blocks. Rootstocks that favour short growth cycles and induce rapid ripening may also minimise the effect of leafroll disease infection on grape quality (Cabaleiro *et al.*, 1999).

New approaches to the control of leafroll disease include cross-protection and genetic manipulation of grapevines. Theoretically, cross-protection can be an efficient means of controlling plant virus disease (Fuchs *et al.,* 1997; Gonsalves and Garnsey, 1989). Plants infected with a mild strain of a virus are protected against more severe strains of that virus. Classical cross-protection has, however, been shown to be ineffective for the control of leafroll disease (Krake, 1993).

2.3.2 MOLECULAR STRATEGIES

The public demand for ecologically acceptable control measures for crop protection has created the need for alternative strategies that are not dependant on the application of chemicals. These strategies revolve around the incorporation of foreign genes into the genome of a crop plant, which can lead to the subsequent control of virus infection. The advantages of genetic engineering to control leafroll disease include: 1) reduced pesticide spray for the control of insect vectors; 2) true-to-type cultivars; 3) decreased virus susceptibility; 4) improved fruit quality. This can be achieved either by using natural resistance genes, pathogenesis related proteins or through pathogen-derived resistance (PDR) (Kavanagh and Spillane, 1995; Lomonossoff, 1995). Natural resistance genes such as the *Tm-1*, *Tm-2* and *Tm-2*^{2} from tomato, *N* and *N'* from tobacco and the R_x and R_y genes from potato and the pathogenesis related proteins involved in the hypersensitive response which activate programmed cell death around the initial site of infection have not been identified in grapevine. Other forms of natural resistance strategies involve the use of broad-spectrum anti-viral genes. The use of a broad-spectrum anti-viral resistance gene, the ribosome-inactivating protein (RIP) from *Dianthin* species is currently being investigated. These proteins modify the ribosomal RNA and interfere with polypeptide translation (Kavanagh and Spillane, 1995).

2.3.2.1 Pathogen derived resistance

This type of resistance is obtained from the expression of genes derived from the pathogen itself. The transformation of plants with viral genes often gives rise to plants that are resistant to the virus from which the gene sequence was derived. The mechanism of action of PDR is diverse and varies with different genes. The general principle of PDR follows that when genes are expressed in plants at an inappropriate concentration, form or at an inappropriate time in the infection cycle, virus infection is inhibited and the pathogenicity of the virus is reduced. Therefore, the incorporation of a virus-derived gene from a grapevine-infecting virus into the grapevine genome may provide resistance against or tolerance to specific grapevine-infecting viruses for which there are no known natural resistance genes.

The first example of this approach was achieved in 1986, when Powell-Abel *et al.* (1986) obtained resistance to *tobacco mosaic tobamovirus* (TMV) in transgenic plants expressing the TMV CP. Since this pioneering experiment, more than 30 viruses have been targeted for control through PDR. Any coding or non-coding sequence of the viral genome can be used for PDR. The most common approach is the use of the CP, but any of the replication (Rep)-associated proteins, the movement protein (MP) or any other non-structural protein can also be used (Lomonossoff, 1995). Coat protein-mediated resistance (CPMR) and replicase-mediated resistance (RepMR) offer very high levels of resistance to homologous viruses, or viruses that are very closely related, but do not offer resistance against unrelated viruses. Movement protein-mediated resistance (MPMR), however, induces low levels of resistance, but this resistance is often effective agains unrelated viruses, thus offering broad-spectrum reistance (Kavanagh and Spillane, 1995).

Pathogen-derived resistance can be mediated either through the accumulation of RNA or through the production of proteins (Baulcombe, 1996). RNA-dependent mechanisms exhibit low mRNA accumulation levels. RNA-mediated virus resistance occurs in the cytoplasm and appears to involve a specific mechanism of post-transcriptional gene silencing (PTGS). PTGS appears to be a natural defence mechanism against RNA sequences derived from pathogens (Covey *et al.*, 1997). PTGS is an RNA degradation mechanism that can be induced by viruses, transgenes or endogenous genes. PTGS results in the sequence specific degradation of RNA, which in turn prevents the accumulation of RNA with homology to the inducing molecule. Double-stranded RNA (dsRNA) triggers the PTGS mechanism. The dsRNA is degraded into 21-25 nt sense and antisense RNA molecules. These short RNA molecules are incorporated into a ribonuclease complex to form a RNA-induced silencing complex (RISC). The active RISC targets the homologous RNA transcript by base pairing and degrades the mRNA (Cerrutti, 2003; Vaucheret *et al.*, 2001) This RNA-dependant resistance results in very high levels of resistance and is specific to the targeted gene.

Protein-dependent mechanisms exhibit high mRNA transcript levels. The mechanism of resistance varies depending on the virus gene used. In general, the

expressed protein interferes with the infection cycle and inhibits a particular process from occurring. An example is the TMV CP, which is thought to prevent the uncoating of the RNA and thus prevents viral replication (Register and Beachy, 1988). Other virus proteins such as the MPs confer resistance when modified forms of the protein are expressed (Cooper *et al.*, 1995). These proteins act in a dominant negative fashion and outcompete the virus encoded MP.

2.3.2.1.1 Movement protein-mediated resistance

MPMR follows a protein-based resistance mechanism. All reported examples of broad-spectrum MPMR have required the accumulation of a dysfunctional MP for a resistance phenotype to develop (Beck *et al.*, 1994; Cooper *et al.,* 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.*, 1997; Tacke *et al.*, 1996). The MPMR strategy is dependent on the expression of a dysfunctional MP that is able to outcompete the wild-type MP in the form of a dominant-negative interaction (Beck *et al.*, 1994; Cooper *et al.,* 1995; Lapidot *et al.*, 1993). Resistance is dependent on the effectiveness of the mutations to inhibit a function of the MP that is required for movement. Seppänen *et al.* (1997) and Tacke *et al.* (1996) showed that levels of resistance were dependent on different types of mutations. Certain mutations in the MP were able to confer resistance, whilst other mutations had no or little effect on virus infection. The expression of a functional MP may also increase the susceptibility of the plant to virus infection (Cooper *et al.*, 1995; Ziegler-Graff *et al.*, 1991). The resistance is also effective against challenge with a virus particle or the virus genome (Beck *et al.*, 1994; Lapidot *et al.*, 1993). Tacke *et al.* (1996) demonstrated that MPMR can follow an RNA mechanism, but this resulted in resistance against the homologous virus only.

MPMR is often observed as a delay in disease symptoms (Cooper *et al.,* 1995; Lapidot *et al.*, 1993), or as a reduction in virus accumulation (Beck *et al.*, 1994; Cooper *et al.,* 1995; Tacke *et al.*, 1996). MPMR is observed against the homologous virus and closely related and unrelated viruses (Beck *et al.*, 1994; Cooper *et al.,* 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.*, 1997; Tacke *et al.*, 1996). The level of resistance is usually low (Cooper *et al.,* 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993), but Tacke *et al.* (1996) reported resistance of between 70 and 80% against unrelated viruses with a dysfunctional MP construct. Resistance is usually observed at low levels of virus challenge, but is overcome by infection with very high levels of virus. The levels of resistance also vary with the different viruses (Cooper *et al.*, 1995; Tacke *et al.*, 1996). Cooper *et al.* (1995) found that resistance against unrelated viruses, was only observed in systemic infection and did not affect the cell-to-cell movement of the viruses in the infected leaf.

2.4 PLANT VIRUS MOVEMENT

In order for a plant virus to infect a susceptible host plant, the virus must move from the site of infection by cell-to-cell movement towards the vascular tissue. Once in the vascular tissue, the virus will move long distances to other tissues to establish a systemic infection and produce a disease. The major barrier to plant virus movement comes in the form of the impermeable cell wall, which acts as a barrier to the release and uptake of progeny viruses by surface fusion and receptor-mediated endocytosis, mechanisms used by animal viruses (Cann, 1997). Because the plant cell wall acts as a barrier to the extracellular release and uptake of plant virus progeny, plant viruses have evolved a mechanism for cell-to-cell movement that evades the cell wall barrier (Atabekov and Dorokhov, 1984). Plant viruses move from cell-to-cell and over long distances by modifying the pre-existing pathways for macromolecular transport provided by the plasmodesmata and vascular tissue (Atabekov and Taliansky, 1990). Most plant viruses encode a movement specific protein. This MP interacts with the plasmodesmata and facilitates the movement of the virus into adjacent cells. The role of the MP in cell-to-cell movement has been determined in a number of diverse plant viruses. Apart from the MP, some other virus encoded proteins may provide additional functions which are required for cell-to-cell and long distance movement (Carrington *et al.*, 1996). It has also been shown that many MPs are genetically interchangeable (DeJong and Ahlquist, 1992). Furthermore, viruses that are deficient in movement in a particular host can be complemented when infected with an unrelated virus that is movement competent in that host (Atabekov and Taliansky, 1990).

Different viruses use different mechanisms to overcome the problem of movement through the plasmodesmata into adjacent cells. Although some principles of virus movement are common amongst the viruses, the nature of movement varies between the virus taxa. Successful movement is also regulated by interactions of the MPs and other viral proteins with the endomembrane system and the cell cytoskeleton network (Lazarowitz and Beachy, 1999).

Tobacco mosaic tobamovirus and *red clover necrotic mosaic dianthovirus* (RCNMV) represent the simplest form of movement for plant viruses. The viruses move as an RNA molecule, the virus codes for a single MP and the CP is not required for cell-to-cell movement. The CP is, however, required for long distance movement. The MP binds ssRNA, increases the size exclusion limit of the plasmodesmata in mesophyll cells and localise to, but does not change the physical appearance of the plasmodesmata (Lucas and Gilbertsen, 1994). *Tobacco mosaic tobamovirus* replicates in the cell in association with the endoplasmic reticulum. The TMV MP binds to the newly synthesised genome to form a long thin MP-RNA complex. The TMV MP also binds to the cell cytoskeleton and targets the MP-RNA complex to the cell wall and plasmodesmata where it binds to the plasmodesmata. However, independent experiments have shown that the cell cytoskeleton is dispensible for cell-to-cell movement of TMV (Gillespie *et al.*, 2002). After binding to

the plasmodesmata, the TMV MP increases the permeability of the plasmodesmata, thus allowing the MP-RNA complex to move through the channel and enter the adjacent host cell (Lucas and Gilbertsen, 1994). The increase in the size exclusion limit caused by the TMV MP is limited to the leading edge of the infection (Oparka *et al.*, 1997).

The *cucumber mosaic cucumovirus* (CMV) require the 3a MP and CP for cell-to-cell movement (Canto *et al.*, 1997). The MP has functions similar to those of the TMV MP: 1) localises to and moves through the plasmodesmata; 2) increase the size exclusion limit of the plasmodesmata; 3) binds ss nucleic acids *in vitro*; 4) complements movement deficient mutants. Although CMV undergoes cell-to-cell movement as an RNA molecule, the CMV MP cannot promote viral cell-to-cell movement without its cognate CP. The mechanism of movement for CMV is unknown, but it is believed that the CP C-terminal domain interacts with the MP or MP-RNA complex to facilitate the cell-to-cell movement of the virus (Nagano *et al.*, 2001).

Viruses of the *tospo-*, *como-*, *caulimo-* and *nepoviruses* code for a single MP, and also require the CP for cell-to-cell movement. These viruses move as intact encapsidated virus particles through tubules, unlike CMV. The tubules appear to be localised at or near modified plasmodesmata that do not contain the central desmotubule. The MP is the only virus encoded protein that is located in the tubule structure, which protrude into adjacent cells. The CP is required for the formation of the virus particles, which move through the tubule to adjacent cells (Kasteel *et al.*, 1996). For *cowpea mosaic comovirus* (CPMV) cell-to-cell movement, the virus moves as an intact virus particle. The CPMV MPs localise to the plasmodesmata and induce the removal of the desmotubule. The CPMV MPs then assemble into the tubule structure and start a unidirectional extension into the adjacent cell. The virus particles assembled in the cytoplasm are escorted to the tubule by the CPMV MPs and the virus particles are transported through the tubule by interactions between the MP and the CP (Wellink and Van Kammen, 1989).

A diverse group of viruses including the *potex-*, *hordei-*, *furo* -and *carlaviruses* encode a cluster of three proteins, the triple gene block (TGB), which are required for cell-to-cell and systemic movement (Carrington *et al.*, 1996). PVX also requires the CP for cell-to-cell and systemic movement (Santa Cruz *et al.*, 1998). The TGBp1 proteins contain an NTPase / helicase domain, which has ss nucleic acid binding ability. The TGBp2 and TGBp3 proteins are membrane-associated proteins that are targeted to the cell periphery and facilitate the movement of the TGBp1 to the plasmodesmata (Yang *et* al., 2000). The PVX CP localises to the plasmodesmata, but has no effect on the size exlusion limit of the plasmodesmata (Santa Cruz *et al.,* 1998). The TGBp1 protein can increase the size exlusion limit (Angell *et al.*, 1996), but is not localised to the plasmodesmata by itself. Rather the TGBp1 is localised to cytoplasmic inclusion bodies (Davies *et al.*, 1993). *Potato X potxvirus* moves as a complex of RNA, CP and TGBp1 through modified plasmodesmata. This complex is

targeted to the plasmodesmata by the TGBp2 and TGBp3 proteins (Lough *et al.*, 1998; Solovyev *et al.*, 2000).

The bipartite geminiviruses encode two MPs for cell-to-cell movement. The CP is not required for either cell-to-cell or long distance movement. The requirement of two MPs is neccesitated by the ssDNA nature of these viruses. They undergo a dsDNA intermediate step during replication, which occurs in the nucleus. The two MPs, movement protein on the B component (MPB) and the nuclear shuttle protein (NSP) have distinct functions but co-operate to move the viral genome (Sanderfoot and Lazarowitz, 1996). The NSP binds to ssDNA (Pascal *et* al., 1994) and the MPB increases the size exclusion limit of the plasmodesmata (Noueiry *et al.*, 1994). The NSP binds to the ssDNA viral genome and shuttles the ssDNA between the nucleus and the cytoplasm. The MPB binds to the NSP-ssDNA complex in the cytoplasm and moves the complex through the cytoplasm to and across the cell wall into adjacent cells. In the new cell, the MPB releases the NSP-ssDNA complex, which is then shuttled back to the nucleus for replication (Sanderfoot and Lazarowitz, 1996).

Members of the *Closteroviridae* encode an Hsp70h protein, which has been shown to act as a MP. The Hsp70h from *beet yellows closterovirus* (BYV) is targeted to the plasmodesmata (Medina *et al.*, 1999), mediates movement of a movement deficient potexvirus (Agranovsky *et al.*, 1998) and is regarded as a MP of BYV (Peremyslov *et al.*, 1999). The cell-to-cell movement of BYV is dependent on the correct assembly of the virus particle. The body of the virus particle is self-assembled by interactions between the CP and the RNA genome. The tail structure, which is required for movement, contains four proteins, the divergent CP (dCP), the Hsp70h, the 64 kDa protein (p64) and the 20 kDa protein (p20) which interacts with the Hsp70h (Dolja, 2003). After virus assembly, BYV can undergo cell-to-cell movement. The 6 kDa (p6) protein, Hsp70h, p64, CP and dCP are all required for BYV movement. In addition, the p20 protein is also required in an accessory role. The ATPase activity of the Hsp70h is believed to provide energy for the movement of the large virus particle. After virus assembly, the intact virus particles are chaperoned toward the plasmodesmata via an interaction between the cell cytoskeleton and the Hsp70h. The Hsp70h anchors to the plasmodesmata and the virus is translocated through the plasmodesmata by a mechanical force generated by the Hsp70h (Alzhanova *et al.*, 2001). The role of the non-structural p6 in virus movement has not yet been determined. This protein has a transmembrane domain, similar to TGBp3 of *potato X potexvirus*. This model explains the cell-to-cell movement of BYV. Other members of the *Closteroviridae,* however, are not efficient in cell-to-cell movement and therefore may not follow the same model of movement.

Plant viruses use different methods of moving from the site of replication to the cell wall and through the plasmodesmata. Although the role of the MPs may vary with the different viruses, their role in virus movement and disease development is essential.

For systemic infection and disease develoment, viruses must undergo cell-to-cell movement through the mesophyll, bundle sheath, phloem parenchyma and companion cells. Long distance movement to other tissues is achieved by movement from the vascular parenchyma and companion cells to the sieve elements, where the viruses undergo bulk flow following the flow of photo-assimilates from source to sink tissues. At the systemic tissues, virus particles enter into the companion cells from the sieve elements and then undergo cell-to-cell movement into bundle sheath, mesophyll and epidermal cells (Carrington *et al.*, 1996).

The long distance movement of plant viruses is less well understood and remains a "black box". The mechanism of virus entry and exit into the phloem system is not known (Santa Cruz, 1999). The vascular tisue is surrounded by bundle sheath cells, which include vascular parenchyma and companion cells. The plasmodesmata connecting these cells are different from the plasmodesmata connecting the mesophyll cells through which viruses undergo cell-to-cell movement (Nelson and van Bel, 1998). Once in the phloem, the viruses are transported by bulk flow following a "source to sink" flow.

The MP is required for specific long-distance movement functions (Fenczik *et al.*, 1995). The entry of virus particles into the plant vascular system and the subsequent systemic spread through the plant, however, requires additional functions, which may be supplied by the CP. The role of the CP in systemic spread is unknown, but it may in conjunction with the MP, increase the permeability of the plasmodesmata at the boundary between the vascular and non-vascular tissue (Ding *et al.*, 1992). However, not all viruses require the CP for long-distance movement (Forster *et al.*, 1992; Petty *et al.*, 1990). Other proteins may also be required for long distance movement of viruses (Lazarowitz and Beachy, 1999).

2.5 *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3*

Grapevine leafroll-associated virus-3 is the type member of the *ampelovirus* genus within the *Closteroviridae* (Martelli *et al.*, 2002). It is the most widespread of the leafroll-associated *Closteroviridae* (Boscia *et al.*, 1995), is consistently associated with grapevine leafroll symptoms and is therefore regarded as a genuine agent for grapevine leafroll disease (Belli, 1995; Boscia *et al.*, 1995; Engelbrecht and Kasdorf, 1990a). *Grapevine leafroll-associated virus-3* has also been associated with grapevine corky bark and Shiraz decline in South Africa (Engelbrecht and Kasdorf, 1990a). *Grapevine leafroll-associated virus-3* is phloem limited and non-mechanically transmissible. It is an economically important virus as it spreads rapidly in vineyards and is transmitted by seven species of mealybug and one species of scale insect (Golino *et al.*, 2000; Martelli, 1993). The only known hosts that it can infect are of the *Vitis* family.

Grapevine leafroll-associated virus-3 can be detected in roots, bark tissue, tendrils, flowers, fruit peduncles and fruit (Teliz *et al.*, 1987) and can be isolated from

the petioles of symptomatic leaves and the bark tissue of diseased grapevines. The highest yields of the virus are obtained from the stem phloem tissue of diseased vines (Hu *et al.*, 1990a). These yields are low when compared with other members of the *Closteroviridae* (Hu *et al.*, 1990a). The difficulty of virus purification seems to be due to the aggregation of virus particles as opposed to virus degradation (Hu *et al.*, 1990a), which is generally experienced with large elongated viruses.

The viral particles are 1800 – 1900 nm in length (Zee *et al.*, 1987) (Fig. 2.3) and the M_r of the coat protein is 43 kDa (Hu *et al.*, 1990a) as determined by SDS-PAGE. This M_r differs from the predicted size of 35 kDa as coded for by the CP open reading frame (ORF) (Ling *et al.*, 1997). A dsRNA of approximately 10 x 10^6 M_r, corresponding to 18.5 kb, has been isolated from GLRaV-3-infected grapevines (Hu *et al.*, 1990a; Saldarelli *et al.*, 1994).

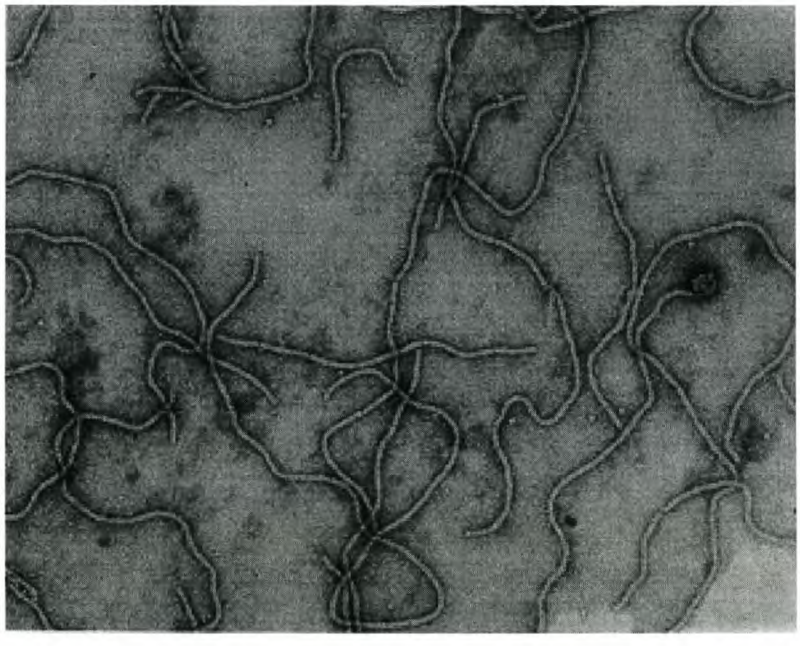

Fig. 2.3 The morphology of GLRaV-3 (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/17010000.htm)

The GLRaV-3 genome has been sequenced (Ling *et al.*, 1998) and contains 16 ORFs, consisting of all the major *Closteroviridae* components. These include: two copies of the leader protease (Pro), a methyl transferase (MTR), a helicase (Hel), an RNA-dependent RNA polymerase, Hsp70h, a heat shock protein 90 kDa homolog (p64), CP and a dCP. The function of the five 3' ORFs has not been determined (Fig 2.4). The genome organisation of GLRaV-3 is consistent with the typical monopartite *Closteroviridae* genome organisation. Viruses of the *ampelovirus* genus differ from those of the *closterovirus* genus in that the *CP* gene is found upstream of the *dCP* gene and the genome contains two copies of the Pro.

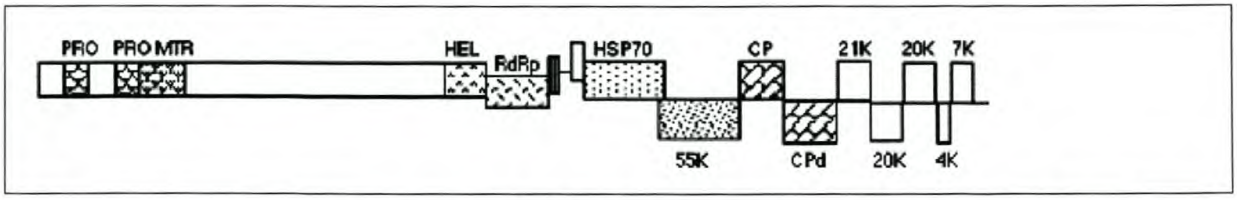

Fig. 2.4 The genome organisation of GLRaV-3, showing the relative position of the 16 ORFs and their expression products. Pro, papain-like protease; MTR, methyltransferase; Hel, helicase; RdRp, RNA-dependent RNA polymerase; Hsp70h, heat-shock protein 70 kDa homolog; CP, coat protein; dCP, divergent coat protein (Martelli *et al.*, 2002).

Phylogenetic analysis of the GLRaV-3 *hel*, *RdRp* and *hsp70h* genes indicates that GLRaV-3 consistently falls into a lineage that is independent from the aphid-transmissible *closteroviruses* and the whitefly-transmissible *criniviruses* (Karasev, 2000) These results have been repeated with the *hsp70h* gene by Fazeli and Rezaian (2000) Ling *et al.* (1998) and Martelli *et al.* (2002). Due to the mixed infection nature of grapevine leafroll and the lack of alternate host plants, very little is known about the biology of GLRaV-3.

2.6 CONCLUSION

The grapevine leafroll-associated viruses are members of the *Closteroviridae.* The role of some of these GLRaVs in leafroll disease has not been established. Some of these viruses have also been associated with other viral diseases of grapevine. More research is required to establish the role of these GLRaVs in leafroll disease and their role in symptom severity and effects on the vine and fruit.

Leafroll disease affects vegetative growth and the quantity and quality of the fruit. These effects are due to the phloem degeneration brought about by virus infection and affect the translocation of photo-assimilates from the leaves to the fruit and growth tips. Current data indicate that GLRaV-1 has an effect on the yield and that GLRaV-3 has an effect on the fruit quality. Due to the detrimental effect of the GLRaVs on vegetative growth and fruit quantity and quality and the lack of long-term data on the effect of GLRaVs on grapevine survival, it is recommended that leafroll disease should not be used as a devigorating agent in grapevines. Viticultural methods that reduce yield or excessive growth should rather be used.

The most pressing problem faced by grape growers with respect to grapevine leafroll disease is the question of disease control. Detection procedures have improved significantly in the last decade. The use of the most sensitive detection techniques may play a significant role in determining the scale of the disease in South African vineyards. Armed with this information, the effectiveness of current management and control strategies can be determined. Current management strategies and the planting of “virus-free” vines have not had the desired effect in controlling the disease. This is predominantly due to the re-infection of newly established vines and the poor understanding of the viruses associated with the disease. At best, these management strategies can be used as a temporary

mechanism to reduce the spread of disease. For long-term control, other approaches, such as the molecular manipulation of the grapevine genome, need to be employed. The most favourable of these approaches is the use of PDR using a dysfunctional MP to control the disease. Because GLRaV-3 is consistently associated with grapevine leafroll disease and is widespread, the Hsp70h MP from this virus can be used in a MPMR for broad-spectrum control of grapevine leafroll disease. Although at present, the levels of resistance obtained by MPMR are inadequate to completely control a complex disease like grapevine leafroll, increased information about MPs and the role of specific domains and amino acids for functionality of the MPs, can lead to focussed mutagenesis stratetgies. New dysfunctional MP constructs could, therefore, lead to improved levels of resistance against related and unrelated viruses associated with grapevine leafroll.

2.7 REFERENCES

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CHAPTER 3

RESEARCH RESULTS

Polymerase chain reaction amplification of the *grapevine leafroll-associated virus-3 hsp70h* gene from various grapevine sources

POLYMERASE CHAIN REACTION AMPLIFICATION OF THE *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3 hsp70h* GENE FROM VARIOUS GRAPEVINE SOURCES

3.1 INTRODUCTION

The *Closteroviridae* represent a group of emerging and re-emerging plant virus pathogens (Karasev, 2000) that cause economically important diseases of numerous crop plants, including sugar beet, citrus and grapevine. The *Closteroviridae* include plant viruses with long flexuous particles and positively stranded RNA genomes of up to 20 kb. The classification of the *Closteroviridae* has been difficult due to the low concentration of the virus in infected plants and the problems associated with isolating sufficient virus particles for proper physicochemical analysis. The family classification has recently changed from being based on the number of genome components to being based on genome organisation and insect transmissibility. The family *Closteroviridae* is currently divided into three genera: the aphid-transmissible *closterovirus*, the mealybug-transmissible *ampelovirus* and the whitefly-transmissible *crinivirus* (Martelli *et al.*, 2002). However, the lack of sufficient biological and sequence data on many members of the *Closteroviridae* and the great diversity within the group has resulted in 11 isolates remaining "tentative" members of the different genera and five viruses remaining unassigned members of the family (Martelli *et al.*, 2002).

The *Closteroviridae* family is unique amongst the viruses as it is the only known one in which the members encode a heat shock protein 70 kDa homolog (Hsp70h) (Martelli *et al.*, 2002). Dolja *et al.* (1994) speculated that the common ancestor of the *Closteroviridae* might have acquired this gene from infected plant cells via recombination with cellular mRNA. The Hsp70h protein has subsequently been shown to be required for virus cell-to-cell movement (Agranovsky *et al.*, 1998; Alzhanova *et al.*, 2000; 2001; Medina *et al.*, 1999; Napuli *et al.*, 2000). The role of a specialised chaperone protein in the movement process could be because of the shear size of the *Closteroviridae* virus particles. Hydrolysis of ATP by the Hsp70h may provide extra energy for the movement of the large particles (Alzhanova, 2001).

Viruses of the *Closteroviridae* associated with grapevines are the most diverse group within the *Closteroviridae* and appear to have members within the *closterovirus* and *ampelovirus* genera. A further four isolates remain unassigned within the family.

Grapevine leafroll disease is one of the three most important viral diseases of grapevine (Martin *et al.*, 2000). Nine members of the *Closteroviridae* have been detected in leafroll-infected grapevines (Alkowni *et al.*, 2002; Castellano *et al.*, 1983; Faoro *et al.*, 1981; Mossop *et al.*, 1985; Namba *et al.*, 1979), but the role of each of these viruses in grapevine leafroll disease is not known. *Grapevine leafroll-*

associated virus-3 (GLRaV-3) has consistently been associated with grapevine leafroll disease (Hu *et al.*, 1990; Zimmerman, 1990) and is thought to be a genuine agent of grapevine leafroll (Boscia *et al.*, 1995). Grapevine leafroll disease has a negative effect on the yield and quality of fruit (Goheen, 1988; Scheu, 1936). The grapevine leafroll-associated viruses (GLRaVs) have not been well characterised due to the inability of the viruses to be mechanically transmitted to herbaceous hosts for amplification and isolation, low virus titres and the high frequency of mixed infection. Double-stranded RNA (dsRNA) is more readily purified from leafroll-infected grapevines than intact virion particles, therefore it is easier to characterise the grapevine-infecting *Closteroviridae* by genome organisation and nucleotide sequence homologies.

The correct and positive identification of the GLRaVs is important for the correct control of the virus and the subsequent disease. This is most evident with GLRaV-2, which is the only member of the aphid-transmissible *closterovirus* genus and may be more closely associated with a rootstock-scion incompatibility disorder than with grapevine leafroll disease. Reports on the identification of GLRaVs isolated from various sources with degenerate primers by RT-PCR have led to the mislabelling of some virus isolates. Saldarelli *et al.* (1998) amplified the partial *hsp70h* gene from five different GLRaV isolates of different grapevine source tissue with a single set of degenerate primers and entered the sequences of these viruses into the public GenBank database. Subsequent sequence analysis (Sefc *et al.*, 2000) and phylogenetic analysis of the *hsp70h* gene (Karasev, 2000) have shown that some of the virus isolates identified by Saldarelli *et al.* (1998) are incorrect. The GLRaV-1 isolate (GenBank accession number Y15890) and the GLRaV-3 isolate (GenBank accession number Y15891) reported by Saldarelli *et al.* (1998) have since been renamed as GLRaV-2 and GLRaV-1 respectively.

We wished to positively identify the GLRaVs used in this project using sequence homology of the intact *hsp70h* gene, amplified from dsRNA isolated from the phloem cortex of three different grapevine sources with distinct leafroll symptoms. Furthermore, a phylogenetic relationship was determined between these new isolates and the grapevine-infecting and other members of the *Closteroviridae*. The Hsp70h was chosen as it is a very useful gene for determining the phylogenetic relationship of the *Closteroviridae*, due to the ease of obtaining these sequences and the availability of these gene sequences from numerous different virus isolates in the GenBank database. Sequence homology and phylogenetic analysis of the three new virus isolates confirmed that GLRaV-3 was isolated from these grapevine sources. One of the grapevine source with which this study was conducted contained a mixed infection and RT-PCR could be used to isolate a specific virus isolate from this mixed infection. All known, complete and partial *Closteroviridae hsp70h* gene sequences were included and the phylogenetic relationships of the GLRaVs based on the *hsp70h* gene sequence are discussed.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL AND PLASMIDS

Vitis vinifera material was collected in the late summer. Cane material for the Stel isolate was obtained from a block of Semillon grapevines grown in a commercial vineyard in Stellenbosch, South Africa. These vines showed clear leafroll symptoms, including the rolling down of the leaf margins, brittleness of the leaf blade and interveinal chlorosis. The mild leafroll isolate (N-1/13/1) and the severe isolate (N-17/14/3) were both obtained from LN-33 vines maintained at the Agricultural Research Council Infruitec-Nietvoorbij (ARC Infruitec-Nietvoorbij) in Stellenbosch, South Africa. These vines were labelled mild and severe according to the symptoms that were produced on the grapevines (Carstens, Pers. comm. Agricultural Research Council – Infruitech-Nietvoorbij, Stellenbosch). They had been maintained at the ARC Infruitec-Nietvoorbij as part of a collection of grapevine leafroll isolates. Cane material was processed immediately or stored at 4°C in paper packages for a maximum period of six months. The pGEM T-easy plasmid vector used for cloning and sequencing the RT-PCR products was purchased from Promega. The pBluescript SK (+) plasmid vector used for cloning the cDNA products was purchased from Stratagene.

3.2.2 dsRNA EXTRACTION

Double stranded RNA (dsRNA) was isolated from the bark scrapings of collected canes, essentially according to the method of Rezaian and Krake (1987), using N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) and phenol extraction. Fifteen grams of cortical bark scrapings were finely cut, flash frozen in liquid nitrogen and ground to a fine powder. Ground bark material was re-suspended in four times the volume of extraction buffer (400mM Tris-HCl, pH 8.0, 1.4 M NaCl, 3% CTAB, 20 mM EDTA, 15mM BME). The slurry was kept at 60°C for 10 min before being stirred for a further 10 min. Half a volume of chloroform was added to the slurry, which was stirred for a further 30 min at room temperature. The slurry was subjected to centrifugation at 10000 g for 10 min. The supernatant was retained, ethanol was added to 17% (v/v) and 2.5 g cellulose CF11 powder (Whatman) was added. The mixture was stirred for 30 min, loaded onto a chromatography column (Promega) and allowed to run through. The cellulose was washed with three volumes of STE-17 (100 mM NaCl, 50 mM Tris-HCl, pH 7, 1 mM EDTA, 17% ethanol v/v) and the dsRNA was eluted with one volume of STE (100 mM NaCl, 50 mM Tris-HCl pH 7, 1 mM EDTA). The dsRNA was further purified by adding ethanol to 17% (v/v) and 0.3 g cellulose CF11 powder to the dsRNA solution. This mixture was stirred for a further 30 min, loaded onto a smaller column (Promega), washed with three volumes of STE-17 and the dsRNA was eluted with STE. dsRNA was precipitated with sodium acetate, re-

suspended in water and visualised on a 0.8% TBE agarose gel stained with ethidium bromide (Sambrook *et al.*, 1989).

3.2.3 cDNA SYNTHESIS

Approximately 100 ng of extracted dsRNA was used for cDNA synthesis according to the protocol supplied with the Promega AMV reverse transcriptase. 100 pM random hexanucleotide primers were added to the dsRNA, denatured at 100°C for 10 min and flash cooled on ice for 5 min. First-strand cDNA synthesis was performed in a 20 µl volume containing 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiotreitol, 40 mM sodium pyrophosphate, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 1 mM dGTP and 30 u AMV reverse transcriptase at 42°C for 60 min. Second-strand cDNA was performed in a 100 µl volume containing 40 mM Tris-HCl at pH 7.8, 90 mM KCl, 2.5 mM $MgCl_2$, 2.5mM DTT, 50 µg/ml BSA, 23 u DNA Polymerase I and 0.8 u RNaseH for 60 min at 12°C, followed by 60 min at 22°C and 10 min at 65°C. Two units of T4 DNA polymerase were added and the reaction mixture was incubated for 10 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 20 mM. Protein was removed by phenol chloroform extraction followed by sodium acetate precipitation (Sambrook *et al.*, 1989). cDNA was re-suspended in 5 µl of water, cloned into the *Eco*RV site of pBluescript SK (+) (Stratagene) and ligated overnight at 16°C. The DNA was transformed into competent DH5α (Stratagene) cells and plated on Luria Bertani agar containing 100 µg/l ampicillin, 200 µg/l IPTG and 200 µg/l X-gal. White colonies were picked and screened for inserts.

3.2.4 RT-PCR AMPLIFICATION AND CLONING

One hundred nanograms of freshly isolated dsRNA was used for a two-step RT-PCR reaction. First-strand cDNA was synthesised according to the protocol supplied with the Promega AMV reverse transcriptase. One hundred pM of a reverse oligonucleotide primer (ClosR4) was used for synthesis and was added to the dsRNA, denatured at 100°C for 10 min and flash cooled on ice for 5 min. First-strand cDNA synthesis was carried out in a 20 µl volume containing 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiotreitol, 40 mM sodium pyrophosphate, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 1 mM dGTP and 30 u AMV reverse transcriptase at 42°C for 60 minutes. First-strand cDNA was diluted 1 to 10 and 1 µl was used as a template for PCR amplification with the GLRaV-3 Hsp70h-specific oligonucleotide primers ClosF1 (5'-CCATGGAAGTAGGTATAGATTTTGG-3') and ClosR4 (5'- TTATCCATTCAAAATCGTGTC-3') in a standard PCR reaction with Expand® High Fidelity polymerase (Roche Biochemicals). A standard PCR amplification programme was used. DNA/RNA hybrids were initially denatured at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec. The reaction was performed in the buffer supplied by the manufacturers, with the addition of 15 mM $MgCl_2$, 20 µM of each oligonucleotide primer and 1 mM of

each nucleotide (dATP, dCTP, dGTP, dTTP). The GLRaV-3-specific oligonucleotide primers were designed from the sequence of the NY-1 isolate published by Ling *et al.* (1998). An *Nco*I restriction site was incorporated into the ClosF3 primer to facilitate later cloning and protein expression (Chapter 4) and no modifications were made to the ClosR4 primer. The *Nco*I restriction site is underlined in the oligonucleotide primer sequence. The resulting RT-PCR products were cloned into the pGEM T-easy vector (Promega) for sequence analysis on an ABI 377 automated sequencer using the dideoxy sequencing method (Sanger *et al.*, 1977).

3.2.5 SEQUENCE HOMOLOGY AND PHYLOGENETIC ANALYSIS

The three new South African GLRaV-3 *hsp70h* gene sequences (Mild, Severe and Stel) were compared for similarity with the sequence database of the National Centre for Biotechnology Information, Bethesda, USA using the BLAST algorithm (Altschul *et al.,* 1990). Pairwise analysis of these sequences was performed with ClustalW version 3.73 (Higgins and Sharp, 1988). Full-length and partial sequences of the *hsp70h* gene were extracted from the GenBank database (National Centre for Biotechnology Information, Bethesda, MD) for all known virus isolates belonging to the *Closteroviridae*. These viruses and sequences are listed in Table 3.1. The three new GLRaV-3, 27 full-length *Closteroviridae* and 28 partial *Closteroviridae hsp70h* sequences and the *GRP78* gene sequence from tomato (GenBank accession number L0880) were analysed to generate a phylogenetic tree of the *Closteroviridae* based on the *hsp70h* gene. The *GRP78* gene sequence was included in the analysis as an outgroup. Sequences were subjected to multiple sequence alignment with Clustal W version 3.73 and refined manually. Phylogenetic analysis was assessed after bootstrapping datasets in 1000 replicates using the PAUP® program (version 4.1) (Swofford, 1993). A consensus tree with bootstrap values was generated.

3.3 RESULTS

3.3.1 dsRNA ISOLATION, cDNA SYNTHESIS AND RT-PCR AMPLIFICATION

dsRNA was isolated from the cortical phloem of three different grapevines infected with grapevine leafroll disease. The yields of dsRNA were relatively low, with approximately 100 ng of dsRNA isolated from 15 g of phloem cortex tissue. The dsRNA preparation from the Stel grapevine sample contained two distinct high M_r genomic and four lower M_r subgenomic dsRNA bands (Fig. 3.1a). A single distinct high M_r genomic band and four lower M_r subgenomic bands were detected in the Mild and Severe grapevine samples (Fig. 3.1a).

Table 3.1 A list of the *Closteroviridae hsp70h* gene sequences available from the NCBI PubMed database (Bethesda, MD). The virus isolates are divided between full-length and partial sequences of the gene. Virus accession numbers with an * have been renamed.

Full-length Hsp70h sequences		Partial Hsp70h sequences	
Virus name	Sequence accession no.	Virus name	Sequence accession no.
Beet yellows virus (BYV)	NC_001598	Apricot stem pitting-associated virus (ApSPaV),	AJ305307
Beet yellow stunt virus (BYSV)	U51931	Beet pseudo yellows virus (BPYV)	Y15568 U67447
Citrus tristeza virus (CTV)	NC_001623 AF26065 Y1842 NC_001661 U56902 AB046398	*Cucurbit yellow stunt disorder virus* (CuYSDV)	AF287474 CYV67170
Cucurbit yellow stunt disorder virus (CuYSDV)	AJ439690	*Grapevine leafroll-associated virus-1* (GLRaV-1)	AJ404738 Y15891
Grapevine leafroll-associated virus-1 (GLRaV-1)	AF195822 AF233935	*Grapevine leafroll-associated virus-2* (GLRaV-2)	Y15890*
Grapevine leafroll-associated virus-2 (GLRaV-2)	Y14131 AF039204	Grapevine leafroll-associated virus-4 (GLRaV-4)	AF039553
Grapevine leafroll-associated virus-3 (GLRaV-3)	AF037268	*Grapevine leafroll-associated virus-5* (GLRaV-5	AF039552
Grapevine leafroll-associated virus-5 (GLRaV-5	AF233934	Grapevine leafroll-associated virus-7 (GLRaV-7)	Y15987
Grapevine rootstock stem lesion virus (GRSLV)	AF314061	Grapevine leafroll-associated virus-8 (GLRaV-8)	AF233936
Lettuce infectious yellows virus (LIYV)	NC_003618	Olive latent yellows-associated virus (OLYaV)	OLV18128
Little cherry virus-1 (LChV-1)	NC_001836	Plum bark necrosis and stem pitting-associated virus (PBNSPaV)	AF195501
Little cherry virus-2 (LChV-2)	AF416335	Potato yellow vein virus (PYVV)	AF150984
Olive latent yellows-associated virus (OLYaV)	AJ440010	*Sweet potato chlorotic stunt virus* (SPCSV)	AJ010920 AJ278651 AJ278650 AJ278653 AJ515381 AF260321
Pineapple mealybug wilt-associated virus-1 (PMWaV-1)	AF414119	*Tomato infectious chlorosis virus* (ToChV)	TIN344213 AY048856 AJ344212 AY048855 AF479662 AY048857 U67449
Pineapple mealybug wilt-associated virus-2 (PMWaV-2)	AF283103		
Sweet potato chlorotic stunt virus (SPCSV)	AJ428555		
Tomato chlorosis virus (ToChV)	AF024630		

cDNA was synthesised from the Stel grapevine sample and cloned into the *Eco*RV site of pBluescript SK (+). One hundred white colonies were picked and analysed for large inserts. Most inserts ranged in size between 100 bp and 500 bp. Fifteen inserts of greater than 500 bp were picked and screened with a 500 bp GLRaV-3 Hsp70h internal probe homologous to nucleotides 5528-6009 in the NY-1 sequence (GenBank accession number AF037268). This internal probe was amplified with degenerate primers ClosF1 (5'-GATTTRAARMGKTGGGTWGG-3') and Clos R2 (5'-TTRTCKATRTTC-TACCRCC-3'). Two clones hybridised to the GLRaV-3 Hsp70h probe. Clone HSP1 showed 96% homology to the *hsp70h* gene from the NY-1 GLRaV-3 isolate, nucleotides 5750-6345, published by Ling *et al.* (1998). Clone HSP2 showed 98% homology to a portion of the leader proteinase gene of the grapevine leafroll-associated virus-2, nucleotides 132-421, published by Zhu *et al.* (1998). Contiguous to this DNA fragment, a further 114 nucleotides had 100% homology to the *hsp70h* gene of the NY-1 GLRaV-3 isolate, nucleotides 5913-6026.

RT-PCR amplification of the dsRNA isolated from the three different grapevine samples yielded single amplification products of 1650 bp (Fig. 3.1b), which were cloned into the pGem T-easy vector to yield plasmids pGem-Mild, pGem-Severe and pGem-Stel.

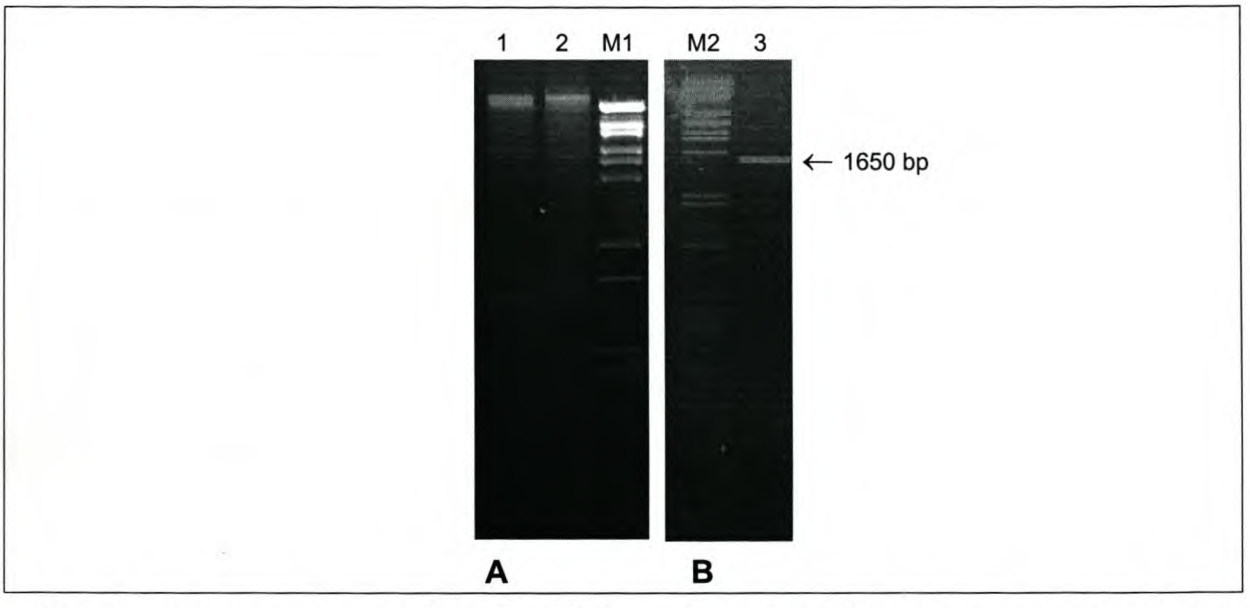


Fig. 3.1 Agarose gels showing the difference in the dsRNA profile of leafroll-infected grapevines Stel and Severe and the size of the RT-PCR product amplified from the Stel grapevine sample. Panel A) Lane 1: dsRNA isolation from the Stel grapevine sample phloem cortex showing two high M_r genomic dsRNA of approximately 20 kb and 18 kb and subgenomic dsRNAs; Lane 2: dsRNA isolation from Severe (N-14/17/3) grapevine sample phloem cortex showing a single high M_r dsRNA band of approximately 18kD and subgenomic dsRNA molecules; Lane M1: λ-*Hind*III marker. Panel B) Lane M2: λ-*Pst*I Marker; Lane 3: 1650 bp RT-PCR product amplified from the dsRNA isolated from the GLRaV-3 Stel sample.

3.3.2 SEQUENCE HOMOLOGY AND PHYLOGENETIC ANALYSIS

The sequences were analysed with the VectorNTI, Version 5.0 suite of sequence analysis software (Informax, Bethesda, MD). The RT-PCR amplification products from the three South African grapevine samples were all derived from GLRaV-3. The sequence homology between the isolates was greater than 94% when compared to the *hsp70h* gene from the NY-1 published sequence. The results of the multiple sequence alignment are shown in Fig. 3.2 and the pairwise homology between the GLRaV-3 *hsp70h* genes and proteins are given in Table 3.2 and Table 3.3 respectively. The Stel and Mild *hsp70h* genes were nearly identical, sharing 99% homology to each other, but only 94% homology to the NY-1 isolate. The mild isolate had 91 and the Stel isolate had 86 nucleotide differences from the NY-1 isolate respectively and shared 82 common nucleotide differences with the NY-1 isolate. The Severe isolate showed 98% homology to the NY-1 isolate, but only 95% homology to the Mild and Stel isolates. The Severe isolate had 25 nucleotide differences from the NY-1 isolate. Fifteen nucleotide differences were common to the three South African isolates. At the amino acid level, the Severe, Stel and Mild isolates had 97, 96 and 96% homology with (Table 3.3) and 11, 18 and 18 amino acid changes from the NY-1 isolate respectively.

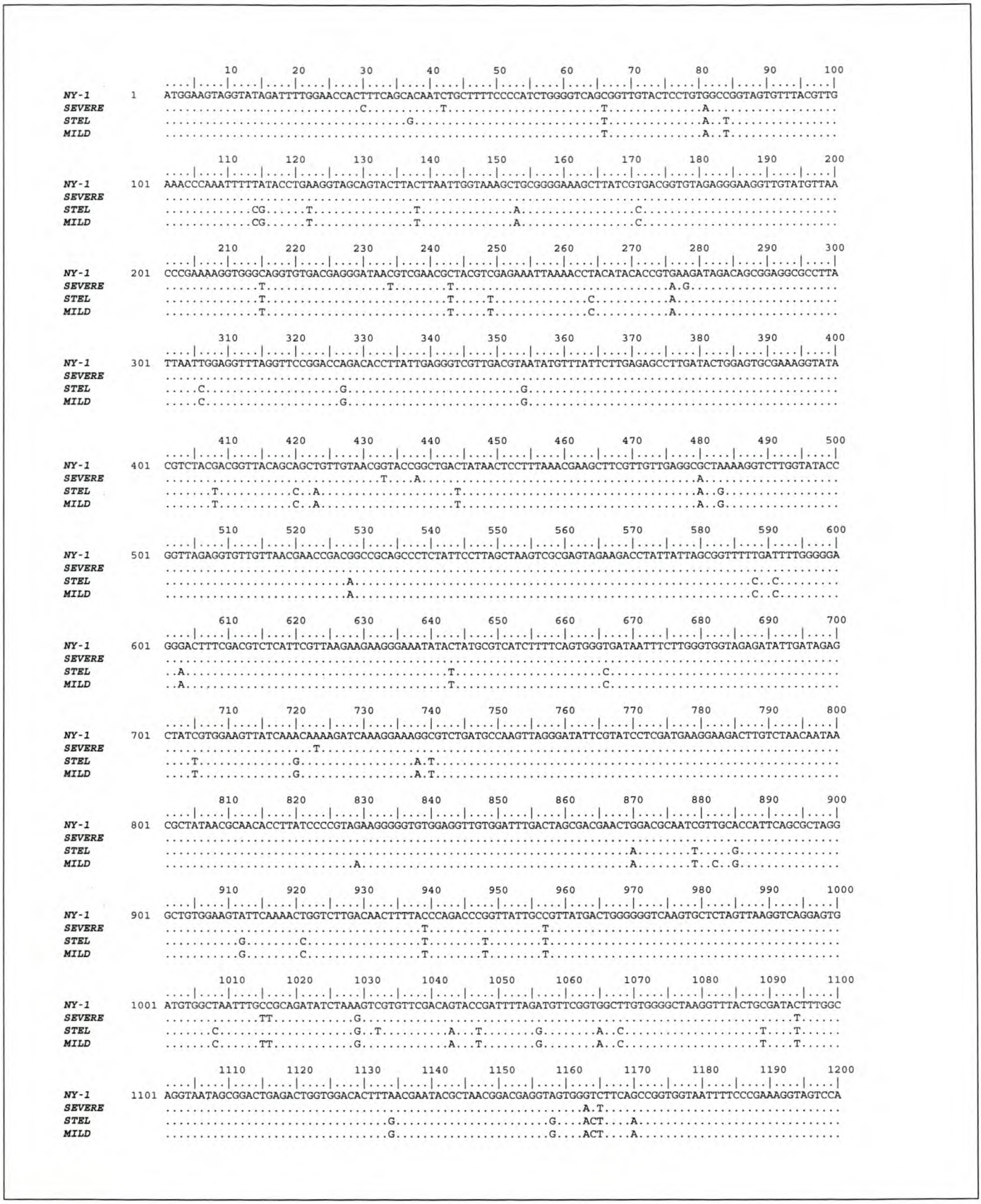
Eighty-four parsimonious trees were produced. One tree was selected and had a tree length of 13124 (Fig 3.3). Phylogenetic analysis of the South African isolates showed that they were located in the *ampelovirus* genus within the *Closteroviridae* and formed a group with the NY-1 isolate. This grouping contained two branches. The Severe isolate grouped with the NY-1 isolate and the Mild and Stel isolates grouped together in a separate branch.

Table 3.2 Pairwise homology between the four GLRaV-3 isolates at the nucleotide level

	Stel	Mild	Severe	NY-1
Stel	-			
Mild	99%	-		
Severe	95%	95%	-	
NY-1	94%	94%	98%	-

Table 3.3 Pairwise homology between the four GLRaV-3 isolates at the amino acid level

	Stel	Mild	Severe	NY-1
Stel	-			
Mild	100%	-		
Severe	97%	96%	-	
NY-1	97%	96%	97%	-



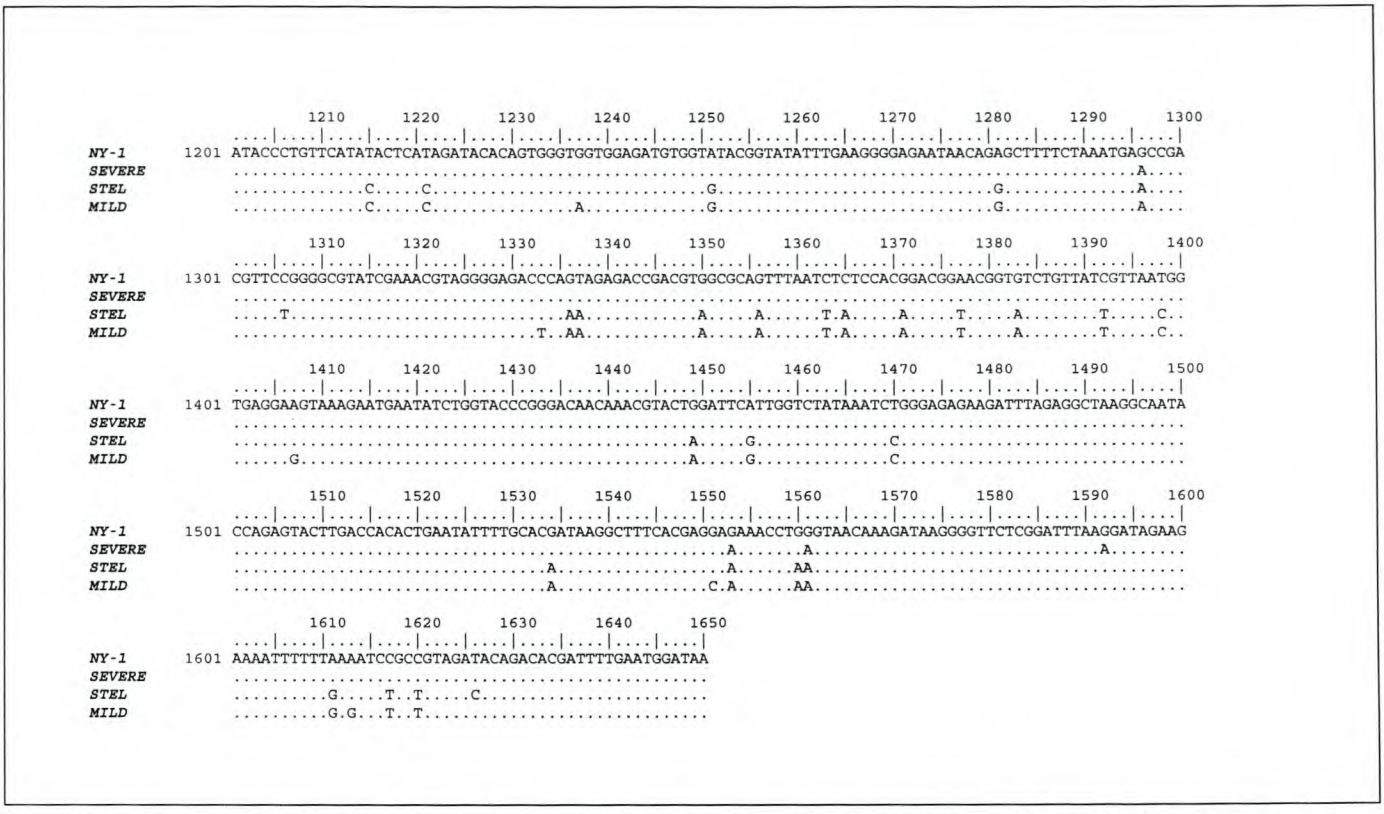


Fig. 3.2 Sequence analysis and multiple sequence alignment of the three GLRaV-3 *hsp70h* genes isolated from South African grapevine sources and compared to the NY-1 published sequence (Ling *et al.*, 1998). The NY-1 sequence is provided in full and only the sequence differences of the three South African isolates are given at the site of the nucleotide change. The Severe isolate contains 25 sequence differences, the Stel isolate contains 86 sequence differences and the Mild isolate contains 91 sequence differences from the NY-1 isolate *hsp70h* gene. Fifteen conserved nucleotide changes are found in the *hsp70h* gene of the South African GLRaV-3 isolates.

3.4 DISCUSSION

It was not possible to isolate an intact, full-length GLRaV-3 Hsp70h cDNA clone from the isolated dsRNA of the Stel isolate. Only 15 clones were found to have inserts larger than 500 bp. Of these inserts, one contained a 604 bp fragment with homology to an internal region of the GLRaV-3 *hsp70h* gene. The second clone contained a 284 bp fragment homologous to a region of the leader protease ORF of GLRaV-2 and a 114 bp fragment homologous to an internal region of the GLRaV-3 *hsp70h* gene. The presence of GLRaV-2 and GLRaV-3 in the Stel isolate was confirmed by ELISA tests performed at the KWV routine services laboratory (results not shown). The difficulties experienced with the cDNA amplification of the GLRaV-3 *hsp70h* gene could have been due to the mixed infection in the grapevine sample used, poor quality of the dsRNA, low yields of dsRNA obtained or inefficient cDNA amplification. Intact *hsp70h* genes were isolated from three different virus sources utilising a single set of GLRaV-3 specific primers.

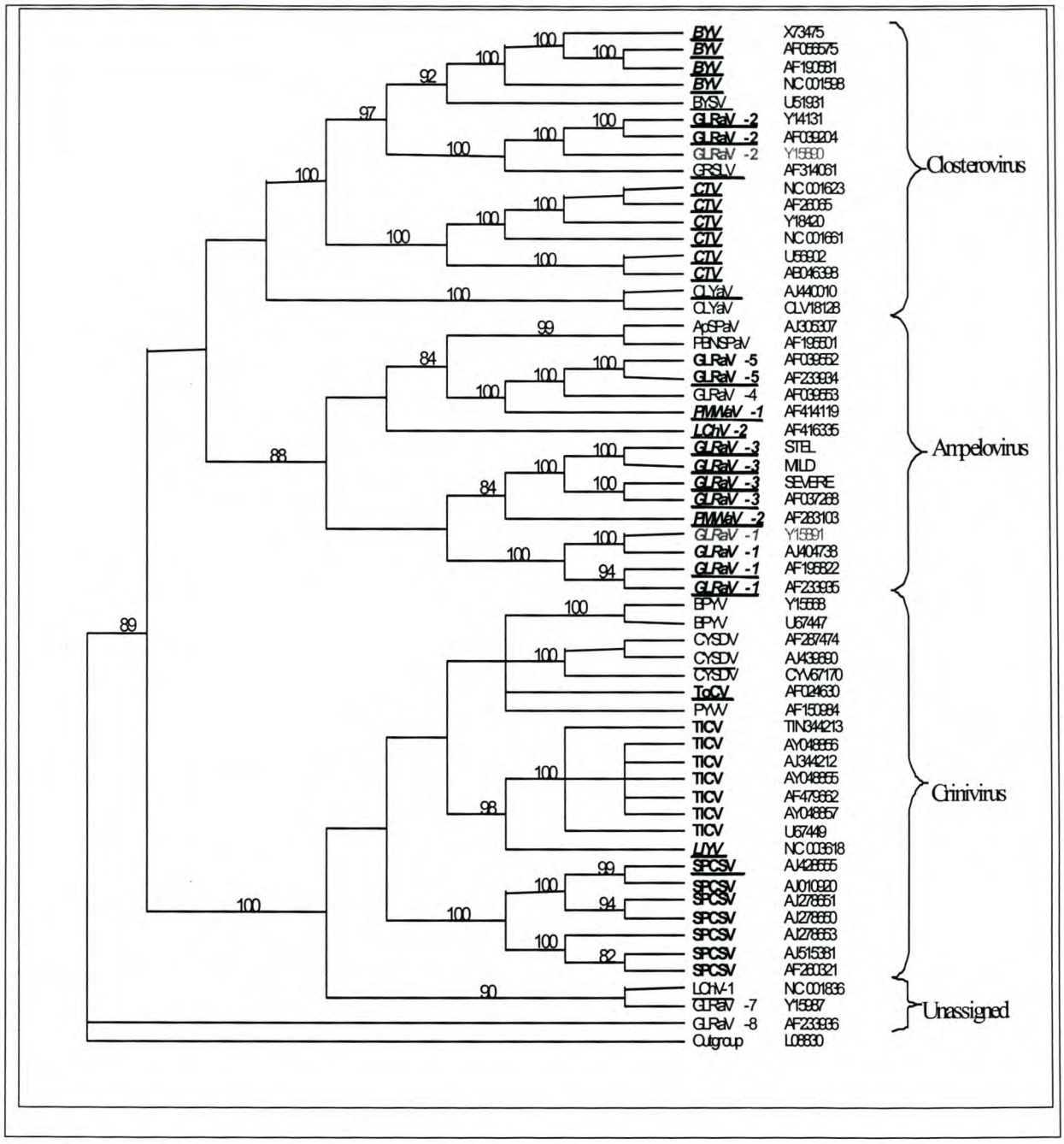


Fig. 3.3 An unrooted phylogenetic tree of the *Closteroviridae* family, based on the *hsp70h* gene. Multiple sequence alignments of 58 *Closteroviridae* partial or complete *hsp70h* gene sequences and one outgroup were aligned with Clustal W. Phylogenetic analysis was performed with PAUP. A representative tree was chosen and subjected to 1000 bootstrap replicates. Nodes with bootstrap support of less than 75% are not indicated, while nodes with bootstrap support above 75% are indicated. Virus sequence accession numbers are indicated after the approved abbreviated virus name. Virus names in **bold** are confirmed members of their respective genera. Virus names that are underlined indicate a full-length *hsp70h* sequence. Virus names in *italic* have proven insect transmission. Virus names in Grey have been reclassified and renamed from their original designation. The outgroup sequence for the alignment is the *GRP78* gene from tomato.

The phylogenetic tree obtained in this work is in general agreement with the tree obtained by Martelli *et al.* (2002), although there are some differences. The differences observed are due to the methods used to draw these trees. Martelli *et al.* (2002) used the Neighbour-Joining method, while the Parsimony method was used in

this study. In order to obtain an accurate phylogenetic tree of the *Closteroviridae*, complete sequences of the *hsp70h* genes are required for analysis. Complete *hsp70h* sequences were not available for all virus isolates. At present, this is the most complete tree of the *Closteroviridae* and is based on all available sequences of the viral *hsp70h* gene. The number of coat protein or replicase gene sequences available in the database are limited, therefore a comparison of this detailed *hsp70h* based tree with a similarly detailed tree based on the *CP* or *Rep* genes cannot be reported at this time.

The phylogenetic tree based on the *hsp70h* gene is consistent with the taxonomic structure of the family and can be used as a general guide to determine the taxonomic relationship of new *Closteroviridae* isolates. The phylogenetic tree is divided into the three distinct genera: the aphid-transmissible *closterovirus*, the mealybug-transmissible *ampelovirus* and the whitefly-transmissible *crinivirus*. From this phylogenetic tree, we have deduced that three viruses are unassigned members of the *Closteroviridae*, viz. GLRaV-7, GLRaV-8 and LChV-1.

The four GLRaV-3 isolates form a group in the *ampelovirus* genus, with two distinct branches. The NY-1 and Severe isolates form one branch and the Mild and Stel isolates form a second branch. This grouping has very good bootstrap support, indicating that there is natural heterogeneity within the GLRaV-3 isolates. The three South African virus isolates are divided between these two branches. It is conceivable that the Severe isolate, which has the highest homology and closest relationship to the NY-1 isolate, was originally imported from the same source as the American NY-1 isolate. Similarly, the Mild and Stel isolates could have derived from the same source, but different from the NY-1/Severe source. Evolutionary pressures in the warm South African environment could have further removed these isolates from the NY-1 isolate, as can be seen by the 15 common nucleotide differences shared amongst the South African isolates.

The GLRaV-3 viruses are most closely related to the *pineapple mealybug wilt-associated virus-2* (PMWaV-2). This finding differs from that of Martelli *et al.* (2002), who reported that GLRaV-3 is most closely related to GLRaV-1. Serological evidence indicates that the GLRaV-1 and GLRaV-3 isolates have a weak serological relationship (Seddas *et al.*, 2000). Our phylogenetic results confirm the close relationship between GLRaV-1 and GLRaV-3, but our data are based on the *hsp70h* rather than the CP.

The *ampelovirus* genus is the largest genus within the *Closteroviridae*, with nine members divided into two distinct clades. The one clade contains the grapevine-infecting viruses GLRaV-4 and GLRaV-5. *Grapevine leafroll-associated virus-5* is a definitive member of the ampelovirus genus and GLRaV-4 is a tentative member. We propose that GLRaV-4 should be included as a definitive member of this genus. The second clade contains the grapevine-infecting viruses GLRaV-1 and GLRaV-3. Unfortunately, the lack of sequence data of the *hsp70h* gene from GLRaV-6 and GLRaV-9 and the availability of only the 3' terminal sequence of GLRaV-8 do not

allow us to assign these viruses as being definitive members of the *ampelovirus* genus. These viruses will therefore remain as tentative members of this genus until further sequence data become available. Sequence analysis of the *hsp70h* gene from the GLRaV-9 isolate indicates that this virus has 79% sequence similarity to GLRaV-5 (Alkowni *et al.*, 2002). However, this sequence was not available in the GenBank database and was therefore not included in this study.

The *closterovirus* genus has six definitive members. These viruses are divided into two distinct clades. *Grapevine leafroll-associated virus-2* forms a distinct branch separate from *beet yellows closterovirus* and *beet yellow stunt closterovirus*. The newly isolated grapevine-infecting *Closteroviridae* isolate, grapevine rootstock stem-lesion virus (GRSLV), is very closely related to GLRaV-2. At present, there are no publications relating to this virus. It is unknown whether this virus is a variant of GLRaV-2 or a new virus isolate. The vector transmissibility of GLRaV-2 and GRSLV has not been determined and biological evidence to support the inclusion of these viruses in the *closterovirus* genus is therefore lacking.

The *crinivirus* genus has seven members. Only partial *hsp70h* gene sequences are available for most of these viruses, which means that there is little bootstrap support for the different branches. A possible fourth grouping, consisting of GLRaV-7 and LChV-1, is located close to the *crinivirus* genus. This branch has 100% bootstrap support, separating it from the crinivirus grouping. The location of this branch within the phylogenetic tree suggests that these two viruses either are distant members of the *crinivirus* genus, or that they could be members of a fourth, as yet unidentified genus within the family *Closteroviridae* and could be transmitted by different insect vectors. The lack of further physical characteristics and information on the insect transmissibility of these two viruses does not yet warrant that they be included in a new genus. We propose that they remain as unassigned members of the *Closteroviridae* until further data become available.

Grapevine leafroll-associated virus-8, a tentative member of the *ampelovirus* genus (Martelli *et al.*, 2002), did not group with any of these three genera and remains unassigned on the basis of *hsp70h* homology. The sequence of the GLRaV-8 *hsp70h* is located in the highly variable C-terminal domain of the gene. Very little homology is found in this region between any of the *Closteroviridae* members (Karasev, 2000) and therefore no phylogenetic relationship between this virus and other members of the *Closteroviridae* could be found.

Sixteen members of the *Closteroviridae* were not included in this phylogenetic study based on the *hsp70h,* as sequence data for these viruses were not available in the GenBank database. Some of these viruses have been included as definitive species in the different genera and some have been included as tentative species in the genera (Martelli *et al.*, 2002).

The phylogenetic analysis shows the heterogeneity of the GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-5. It would be interesting to determine whether the heterogeneity of the GLRaVs will be increased when virus isolates from Africa,

Australia, Europe, North and South America are compared with each other. The phylogenetic tree obtained from the *hsp70h* gene shows that the diversity of the grapevine-infecting viruses of the *Closteroviridae* is much greater than the other members of the *Closteroviridae*. This is to be expected due to the worldwide distribution and the vegetative propagation of the grapevine.

The sequences of GLRaV-1 (GenBank accession number Y15890) and GLRaV-3 (GenBank accession number Y15891) obtained by Saldarelli *et al.* (1998) have previously been shown to be incorrectly labelled (Sefc *et al.*, 2000). This error could have been the result of undetected mixed infections in the original vines, or incorrectly classified virus isolates. Further consideration therefore needs to be given to the three other viruses for which Saldarelli reported to have isolated the partial *hsp70h* gene. This shows an inherent weakness in the use of RT-PCR with degenerate oligonucleotide primers for the identification of new virus isolates. The reported sequence homology of the *hsp70h* gene between GLRaV-4 (GenBank accession number AF039553) and GLRaV-5 (GenBank accession number AF039552) is 91% (Saldarelli *et al.*, 1998). Because of the high homology of the *hsp70h* gene between these two isolates and the close phylogenetic relationship, it is conceivable that these two isolates reported by Saldarelli are variants of a single virus, as seen with the GLRaV-3 isolates presented in this study.

Because of the difficulty of isolation and the lack of biological characterisation of the GLRaVs, taxonomic and phylogenetic studies of these viruses are more easily based on the nucleotide sequences. The *hsp70h* gene is conserved amongst the *Closteroviridae* and regions of the gene share high levels of homology. It is therefore a good candidate for these phylogenetic studies. However, taxonomic studies based on this gene and other isolated genes will continue to change as more biological information and sequence data are obtained for the different virus isolates. The taxonomic state of the *Closteroviridae* will therefore continue to change as more information and sequence data become available.

It is concluded that the three grapevine samples used in these experiments contain definitive GLRaV-3 isolates and that RT-PCR with virus-specific oligonucleotide primers can be used to amplify a specific virus from a mixture of closely related viruses associated with a particular disease. Sequence heterogeneity in the *hsp70h* gene exists amongst the GLRaV-3 isolates, indicating genetic drift within this group of viruses as is seen with many other members of the *Closteroviridae*.

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CHAPTER 4

RESEARCH RESULTS

The mutagenesis and bacterial expression of the Hsp70h from *grapevine leafroll-associated virus-3*

THE MUTAGENESIS AND BACTERIAL EXPRESSION OF THE Hsp70h FROM *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3*

4.1 INTRODUCTION

The 70 kDa heat shock proteins (Hsp70) are molecular chaperones that are found in all cellular organisms. These Hsp70s help the cell to survive under conditions of stress (Lewis and Pelham, 1985). They facilitate protein folding, the assembly of multiprotein complexes, translocation of proteins between cellular compartments and protein degradation (Bukau and Horwich, 1998; Ellis and Hartl, 1999). The Hsp70s have a highly conserved N-terminal ATPase domain (Flaherty *et al.*, 1990) and a variable C-terminal domain (Zhu *et al.*, 1996) that has polypeptide-binding activity.

Crystallographic and homology studies revealed that the active site of the ATPase domain contains conserved acidic amino acids that may take part in ATP hydrolysis (Deluca-Flaherty *et al.*, 1988; Flaherty *et al.,* 1990). For the bovine constitutive cytoplasmic 70 kDa heat shock cognate protein (Hsc70), the acidic amino acids, Asp^{10}, Glu^{175} and Asp^{199}, are required for the correct positioning of the Mg-ATP, and the Asp^{206} acidic amino acid may be the catalytic base required for ATP hydrolysis (Wilbanks *et al.*, 1994). The mammalian immunoglobulin-binding protein (BiP) 70 kDa heat shock protein requires Thr^{37}, Thr^{229} and Glu^{201} for ATPase activity (Gaut and Hendershot, 1993). Single mutations of either of these amino acids resulted in decreased ATPase activity (Gaut and Hendershot, 1993; Wilbanks *et al.*, 1994). Residues that had the greatest effect on ATPase activity were the Asp^{10} and Asp^{199} residues, which are required for ligating the Mg^{2+} ion and positioning the ATP for hydrolysis (Wilbanks *et al.*, 1994).

Molecular chaperones were originally discovered because of their role in bacteriophage λ replication in *Escherichia coli* (Saito and Uchida, 1978; Yochem *et al.*, 1978) whereby it was discovered that the bacterial Hsp70, DnaK, is required for the assembly of the bacteriophage replication machinery (Georgopoulos *et al.,* 1990). It has since been found that a number of bacterial, plant and animal viruses rely on the multiple functions of the Hsp70s (Sullivan and Pipas, 2001). Hsp70s facilitate adenovirus replication (Glotzer *et al.,* 2000), *hepadnavirus* assembly and reverse transcription (Hu *et al.*, 1997), influenza (Gething *et al.*, 1986) and HIV (Knarr *et al.*, 1999) glycoprotein trafficking and *polyomavirus* assembly (Cripe *et al.*, 1995). Plant and animal viruses may also activate the expression of cellular Hsp70s (Aranda *et al.*, 1996; Phillips *et al.*, 1991) or recruit Hsp70s via interactions with the virus-encoded J-domains (Kelley, 1999). Members of the *Closteroviridae* are the only known viruses that encode a virus-specific heat shock protein 70 kDa homolog (Hsp70h) (Martelli *et al.*, 2002).

Virtually all plant viruses actively move to adjacent cells via the plasmodesmata during infection. Cell-to-cell movement is an active process requiring the function of virus-coded movement proteins (MP) (Carrington *et al.*, 1996). The MPs are able to modify the plasmodesmata, thereby increasing the size exclusion limit to allow larger viral macromolecules to pass through. The Hsp70h from the type member of the *Closteroviridae*, *beet yellows closterovirus* (BYV), functions as a MP (Peremyslov *et al.*, 1999). The role of the BYV Hsp70h in movement is to facilitate the assembly of the virion (Alzhanova *et al.,* 2001), the translocation of the virion along the cell cytoskeleton (Karasev *et al.*, 1992) to the plasmodesmata, the anchoring of the virion complex within the plasmodesmal channel (Medina *et al.*, 1999) and translocation through the plasmodesmal channel (Alzhanova *et al.,* 2001). The requirement for a specific chaperone as a MP may be due to the sheer size of the BYV particle, which may need extra energy that is provided by the hydrolysis of ATP by the Hsp70h-mediated ATPase domain (Alzhanova *et al.*, 2001).

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Bos taurus      1   MAKNMAIGIDLGTTYSCVGVFQHGKVEIIA 30
E coli DnaK     1   --MGKIIGIDLGTTNSCVAIMDGTTPRVLE 30
Homo sapiens    1   --KAAAIGIDLGTTYSCVGVFQHGKVEIIA 30
BYV             1   ---MVVFGLDFGTTFSSVCAYVGEELYLFK 30
GLRaV-3 NY1     1   ----MEVGIDFGTTFSTICFSPSGVSGCTP 30
GLRaV Stel      1   ----MEVGIDFGTTFSAICFSPSGVSGCTP 30

Bos taurus      163  GVIAGLNVLRIINEPTAAAIAYGLDRT 188
E coli DnaK     158  GRIAGLEVKRIINEPTAAALAYGLDKG 184
Homo sapiens    160  GVIAGLNVLRIINEPTAAAIAYGLDRT 186
BYV             167  VNLSGYPCVYMVNEPSAAA-LSACSRI 193
GLRaV-3 NY1     161  LKGLGIPVRGVVNEPTAAA-LYSLAKS 186
GLRaV Stel      161  LKGLGIPVRGVVNEPTAAA-LYSLAKS 186

Bos taurus      189 GKGERNVLIFDLGGGTFDVSILTIDDG 227
E coli DnaK     184 -TGNRTIAVYDLGGGTFDISIIEIDEV 209
Homo sapiens    187 GKGERNVLIFDLGGGTFDVSILTIDDG 213
BYV             194 KGATSPVLVYDFGGGTFDVSVISALNN 220
GLRaV-3 NY1     187 RVEDLLLAVFDFGGGTFDVSFVKKKGN 213
GLRaV Stel      187 RVEDLLLAVFDFGGGTFDVSFVKKKGN 213
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Fig. 4.1 A comparison of peptide sequences showing the conserved sequences located in the N-terminal ATPase domain of various Hsp70 proteins. Conserved sequences that are required for ATPase activity are highlighted in **bold**. The GenBank accession numbers of the sequences are: Bos Taurus, AAA73914; *E. coli* DnaK, P04475; *Homo sapiens* ATPase domain, 1HJOA; BYV, CAA37551; GLRaV-3 NY-1 AAC40708. The sequence for GLRaV-3 Stel is given in Chapter 3.

The acidic amino acids of the cellular BiP, DnaK and Hsc70 proteins are also conserved within the *Closteroviridae* Hsp70 proteins (Fig. 4.1). The conserved Asp^{7} and Glu^{181} acidic amino acids are essential for movement of BYV. When these amino acids are mutated, intercellular translocation of the BYV is abolished (Alzhanova *et al.*, 2001; Peremyslov *et al.*, 1999).

To obtain movement protein-mediated resistance, a dysfunctional homologue of the virus MP is required for expression in the plant. Site-directed mutagenesis is a powerful tool to incorporate specific mutations within a particular gene (Piechocki and

Hines, 1994). Four mutations were incorporated into the *grapevine leafroll-associated virus-3* Hsp70h protein by site-directed mutagenesis (Deng and Nikoloff, 1992). These mutations corresponded to the conserved amino acids that previously were shown to be required for ATPase activity (Gaut and Hendershot, 1993; Wilbanks *et al.*, 1994). Two of these mutations in the BYV Hsp70h abolish movement and translocation of the virus in plants (Alzhanova *et al.*, 2001; Peremyslov *et al.*, 1999). However, no biochemical tests were performed on the ATPase activity of the BYV Hsp70h or its mutated homologues.

In this chapter, the mutagenesis of the GLRaV-3 Hsp70h protein and attempts to obtain active proteins for biochemical tests on the ATPase activity of the WT-Hsp and Mut-Hsp proteins of GLRaV-3 are discussed.

4.2 MATERIALS AND METHODS

4.2.1 RT-PCR AMPLIFICATION AND CLONING

The *hsp70h* gene from *grapevine leafroll-associated virus-3* (GLRaV-3) was amplified with oligonucleotide primers Clos F3 and Clos R4 (Table 4.1) from dsRNA isolated from the Stel grapevine sample (Chapter 3). The C-terminal domain of the gene was amplified with oligonucleotide primers HSP-3 and ClosR4 (Table 4.1) from the cloned GLRaV-3 *hsp70h* gene (pGEM-Stel). The amplification was performed using Expand® High Fidelity enzyme (Roche Biochemicals) in a standard PCR reaction. A standard PCR amplification programme with an initial denaturation step of 94°C, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final elongation of 72°C for 5 min. The reaction was performed in the buffer supplied by the manufacturer with the addition of 15 mM $MgCl_2$, 20 μM of each oligonucleotide primer and 1 mM of each nucleotide (dATP, dCTP, dGTP, dTTP).

The PCR product encoding the C-terminal domain of the Hsp70h was cloned into pGem T-Easy (Promega). The gene was excised with *Nco*I and *Bam*HI, cloned into the corresponding sites of bacterial expression vector pET14b (Novagen) and named pET-ΔHsp3. The orientation of the *ΔHsp3* C-terminal fragment was confirmed by restriction enzyme digestion. The *wt-hsp* and *mut-hsp* genes were excised from the pAlter-WT and pAlter-Mut plasmid vectors with *Nco*I and *Bam*HI respectively and cloned into the *Nco*I and *Bam*HI sites of pET14b. The resulting plasmids, pET-WT and pET-Mut, contained the *wt-hsp* and *mut-hsp* genes respectively, driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter. This cloning strategy removed the 6 x Histidine tag located at the N-terminal end of the protein. Clones were confirmed by restriction enzyme digestion. The pET-ΔHsp3, pET-WT and pET-Mut plasmids were transformed into competent DH5α (Stratagene) cells for nucleic acid manipulation, followed by transformation into competent BL21 (DE3) pLysS cells (Novagen) for protein expression.

4.2.2 SITE-DIRECTED MUTAGENESIS

Conserved amino acids, which are required for ATPase activity in a number of different Hsp70 proteins (Fig. 4.1.), were mutated using site-directed mutagenesis. Three mutagenic primers (Table 4.1) carrying four point mutations were incorporated into the GLRaV-3 *hsp70h* gene. Primer Hsp-Mut1 incorporated two point mutations, a transitional mutation, G → A, conferring an Asp^{6} → Asn point mutation, and a transversional mutation A → C, conferring a Thr^{9} → Pro point mutation. Primer Hsp-Mut2 incorporated a transversional mutation, G → C, conferring a Glu^{174} → Gln point mutation. Primer Hsp-Mut3 incorporated a transitional mutation, G → A, conferring an Asp^{197} → Asn point mutation. Oligonucleotide primers were designed according to the method described by Piechocki and Hines (1994).

Table 4.1 Oligonucleotide sequences required for cloning the grapevine leafroll-associated virus-3 *hsp70h* gene and the C-terminal domain of the gene and for site-directed mutagenesis. Underlined nucleotides in ClosF1 and Hsp-3 are an inserted *Nco*I restriction site and the ATG start codon is indicated in *italics*. The point mutations engineered in the *hsp70h* gene are indicated in **bold** for primers Hsp-Mut1, Hsp-Mut2 and Hsp-Mut3.

Primer	Sequence
ClosF1	CC*ATGG*AAGTAGGTATAGATTTTGG-
ClosR2	TTATCCATTCAAAATCGTGTC
Hsp-3	CC*ATGG*CCAATTTGTTGCAG
Hsp-Mut1	GGAAGTAGGTATA**A**ATTTTGGA**C**CCACTTTCAGCAC
Hsp-Mut2	GGTGTTGTTAAC**C**AACCGAGAGCC
Hsp-Mut3	TAGCGGTTTTC**A**ACTTTGGG

Site-directed mutagenesis was performed as per the manufacturer's instructions (Promega). Essentially, ssDNA was isolated from bacteriophage R408-infected cells. The annealing reaction contained 0.05 pmol of ssDNA, 1.3 pmol of each of the three mutagenic primers (Hsp-Mut1, Hsp-Mut2 and Hsp-Mut3) and 0.25 pmol of each of the marker primers (Amp^{repair}, $Tet^{K/O}$) in a 20 µl volume. The annealing mixture was heated to 75°C for 5 min and allowed to cool slowly to room temperature. DNA was synthesised in 30 µl containing 1 x synthesis buffer, 5 u T_4 DNA Polymerase and 3 u T_4 DNA ligase. The synthesis reaction mixture was incubated at 37°C for 60 min. A further 5 mmol of dNTPs were added and the mixture was incubated at 37°C for another 30 min. Five µl of the mutagenic reaction was transformed into competent ES1301 *mut*S cells (Promega) and grown overnight for DNA isolation. The isolated DNA was transformed into competent JM109 cells (Promega) for further DNA isolation and nucleotide sequencing.

4.2.3 PROTEIN EXPRESSION

Ten millilitres of an overnight culture containing either pET-ΔHsp3, pET-WT or pET-Mut was inoculated into 500 ml of TB medium (12 g/l tryptone, 24 g/l yeast extract,

0.0004 % glycerol, 0.01 M KPO_4) containing 1% glucose, 100 μg/ml ampicillin and 34 μg/mg of chloramphenicol. The cultures were grown for approximately 3 h to an OD_{600} of 0.6. Protein expression was induced with 0.1 or 0.4 mM IPTG. Cells induced with 0.1 mM IPTG were grown for a further 4 h at 30°C, and cells induced with 0.4 mM IPTG were grown for a further 3 h at 37°C. Cells were harvested for protein purification by centrifugation.

4.2.4 ANTIBODY PRODUCTION

Total protein from bacteria expressing ΔHsp3 was denatured and run on a 12% polyacrylamide gel (Laemmli, 1970). The gels were stained with Coomassie brilliant blue (0.5% in water) and the protein bands were excised. Gel slices were homogenised and the protein was purified by electro-elution with a Biotrap BT1000 apparatus (Schleicher and Schuell). N-Lauroylsarcosine was removed by dialysis and the protein was concentrated. Antibodies were prepared by Prof. D.U. Bellstedt of the Department of Biochemistry, University of Stellenbosch according to the method described by Cilliers *et al.* (1989).

4.2.5 PROTEIN PURIFICATION AND REFOLDING

The protein solubilisation and refolding strategy was adapted from Burgess (1996), using the mild anionic detergent, Sarkosyl. WT-Hsp and Mut-Hsp proteins were purified from BL21 (DE3) pLysS bacteria transformed with pET-WT or pET-Mut plasmids respectively, induced with 0.4 mM IPTG and grown at 37°C. Cells were harvested by centrifugation (5 000 g for 5 min) and re-suspended in 1/10 volume TEN50 buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl) containing 10% glycerol, 0.2 mM dithiothreitol (DTT) and Complete® protease inhibitor (Roche Biochemicals). The cells were disrupted by sonication and clarified by centrifugation (10 000 g for 20 min). The supernatant was stored for analysis of the soluble proteins and the pellet containing inclusion bodies was processed further. The inclusion bodies were re-suspended and washed three times with TEN50 buffer containing 2% sodium deoxycholate to remove cellular debris. Inclusion bodies were solubilised in 1/10 volume TEN50 buffer containing 0.3% Sarkosyl, 2 mM DTT and 10% glycerol for 30 min. The solution was clarified by centrifugation (10 000 g for 5 min). The solubilised inclusion bodies were dialysed against 5 l of TEN50 buffer containing 2 mM DTT and 1 mM MgCl for 8 h, followed by dialysis against TEN50 containing 1 mM MgCl for a further 8 h. Proteins were clarified by centrifugation at 10000 g for 30 min.

4.2.6 DEAE ANION EXCHANGE CHROMATOGRAPHY

Refolded proteins were further purified by anion exchange chromatography using a Bio-Rad Econo Chromatography system (Bio-Rad) on diethylaminoethyl (DEAE) fast flow sepharose (Pharmacia). Refolded proteins were loaded onto the DEAE

sepharose column at a flow rate of 1 ml/min. The column was washed with TEN50 until a baseline was reached. Proteins were initially eluted with a step gradient of 5% TEN1000 (20 mM Tris, pH 8.0, 1 mM EDTA, 1000 mM NaCl) from 0 to 50% TEN1000 at a flow rate of 2 ml/min. A final elution regimen included a wash step with 14% TEN1000, followed by a 2% step gradient from 16 to 26% TEN1000, with a final wash of 100% TEN1000.

4.2.7 PROTEIN QUANTIFICATION AND DETECTION

Protein concentrations were determined by the method of Bradford (1976) using a Bio-Rad protein assay kit. Bovine serum albumin was used to draw a standard curve. SDS-PAGE was performed as per Laemmli (1970) on 10 or 12% polyacrylamide gels. Gels were stained with 1% Coomassie PAGE blue (BDH) or silver stained as described by Blum *et al.* (1987). For western blot analysis, gels were electrophoretically transferred to Hybond C-extra nylon membranes (Amersham Pharmacia Biotech) as described by Towbin *et al.* (1979). The antibody raised against the expressed C-terminal domain of the GLRaV-3 Hsp70h was used to detect antigen. The antibody-antigen complex was detected with the ECL detection system (Amersham Pharmacia Biotech).

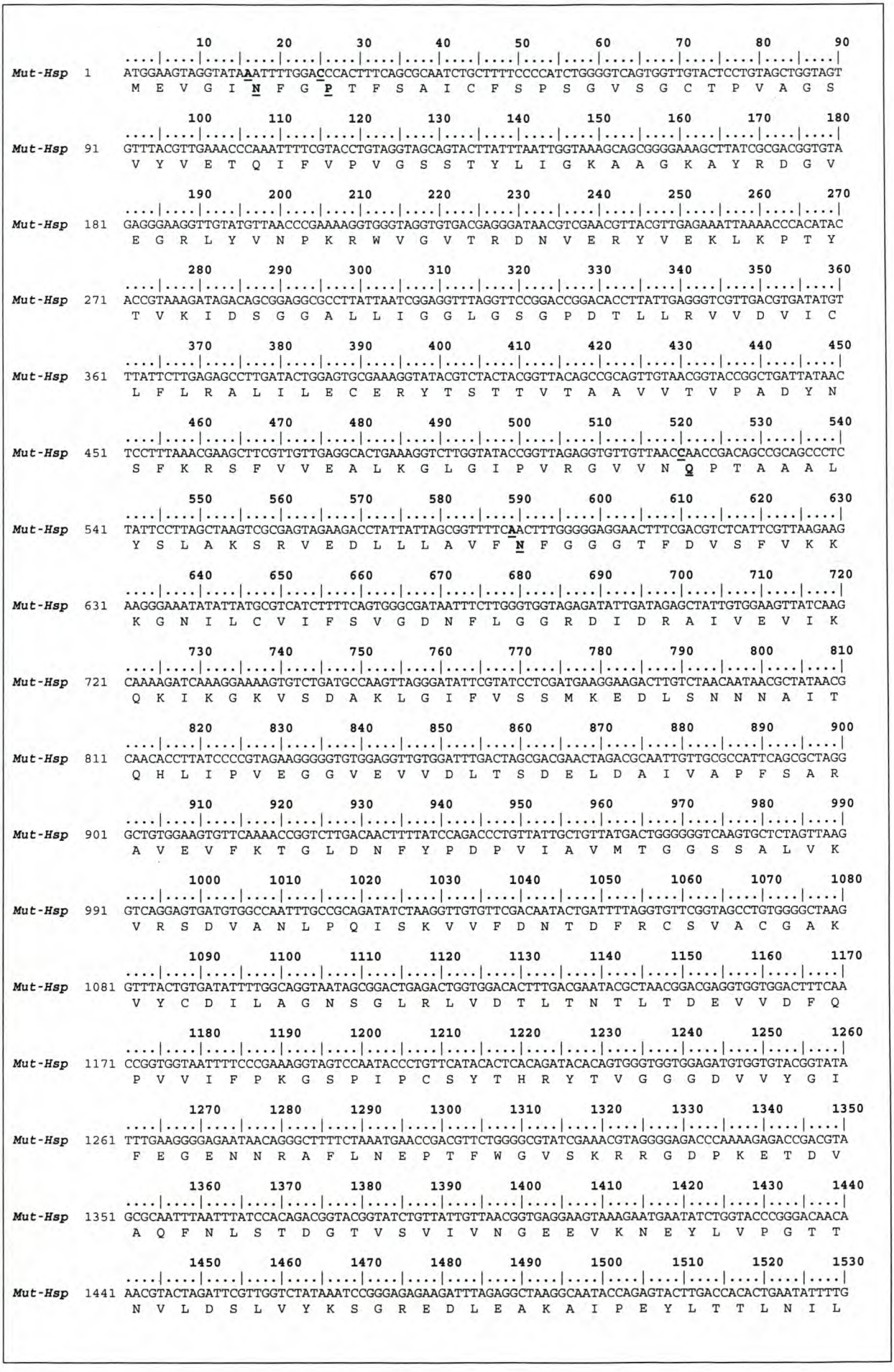
4.3 RESULTS

4.3.1 SITE-DIRECTED MUTAGENESIS

Four point mutations were incorporated in the *wt-hsp* gene to give rise to *mut-hsp*. These mutations were confirmed by the dideoxy sequencing method (Sanger *et al.*, 1977) using an ABI 377 automated sequencer. The point mutations conferred the expected amino acid changes in the protein (Fig. 4.2).

4.3.2 PROTEIN EXPRESSION

Proteins were expressed from BL21 (DE3) pLysS bacteria transformed with the pET-ΔHsp3, pET-WT or pET-Mut plasmids. The ΔHsp3 protein was expressed with 0.4 mM IPTG at 37°C (Fig. 4.3). The expression patterns for WT-Hsp and Mut-Hsp were identical (Fig. 4.4). Expressed proteins from pET-WT and pET-Mut were found in the insoluble fraction of the cells, regardless of the induction procedure. Cells induced with 0.4 mM IPTG expressed the WT- and Mut-Hsp proteins in the insoluble fraction (Fig 4.4). Cells induced with 0.1 mM IPTG and grown at 30°C expressed little WT- and Mut-Hsp proteins in the soluble fraction, as most of the expressed proteins were located in the insoluble fraction (Fig. 4.5).



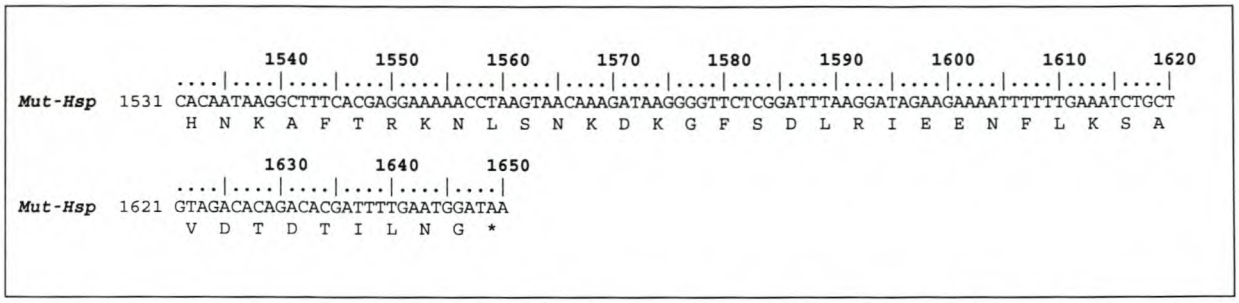


Fig. 4.2 Nucleotide sequence of the Mutated form of the GLRaV-3 Stel *Hsp70h* gene. The mutated nucleotide and amino acid residues are indicated in **bold** and are underlined.

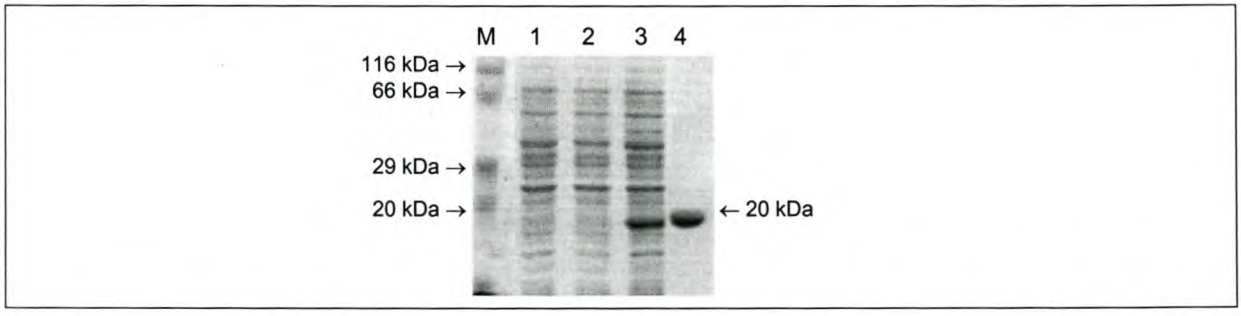


Fig. 4.3 A 12% SDS-PAGE gel showing expression of the ΔHsp3 protein from bacteria BL21 (DE3) containing plasmid pET-ΔHsp3. The gel was stained with 1% Coomassie PAGE blue. Lane M, Rainbow marker (Bio-Rad); Lane 1, total cell protein of BL21 (DE3) pLysS bacteria grown as control, induced with 0.4 mM IPTG and grown at 37°C; Lane 2, total cell protein of BL21 (DE3) pLysS (pET-ΔHsp3) bacteria grown at 37°C, uninduced; Lane 3, total cell protein of BL21 (DE3) pLysS (pET-ΔHsp3) bacteria, induced with 0.4 mM IPTG and grown at 37°C; Lane 4, purified ΔHsp3 protein used for antibody production.

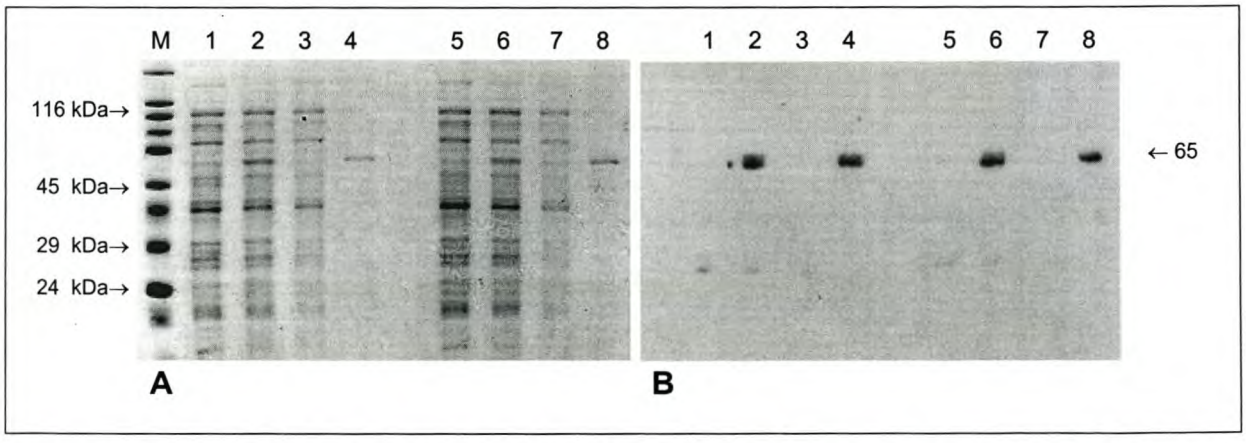


Fig. 4.4 A 10% SDS-PAGE gel showing total cell protein, soluble and insoluble fraction of BL21 (DE3) pLysS bacterial cells containing the pET-WT or pET-Mut plasmids grown at 37°C. Panel A) SDS-PAGE, stained with Coomassie PAGE blue; Panel B) western blot detected with the ECL detection system. Lane M, Wide-range protein marker (Sigma); Lane 1, total cell protein of BL21 (DE3) pLysS (pET-WT) bacteria, uninduced; Lane 2, total cell protein of BL21 (DE3) pLysS (pET-WT) bacteria, induced with 0.4 mM IPTG; Lane 3, soluble proteins isolated from BL21 (DE3) pLysS (pET-WT) bacteria, induced with 0.4 mM IPTG; Lane 4, insoluble protein isolated from BL21 (DE3) pLysS (pET-WT) bacteria, induced with 0.4 mM IPTG; Lane 5, total cell protein of BL21 (DE3) pLysS (pET-Mut) bacteria, uninduced; Lane 6, total cell protein of BL21 (DE3) pLysS (pET-Mut) bacteria induced with 0.4 mM IPTG; Lane 7, soluble proteins isolated from BL21 (DE3) pLysS (pET-Mut) bacteria induced with 0.4 mM IPTG; Lane 8, insoluble protein isolated from BL21 (DE3) pLysS (pET-Mut) bacteria induced with 0.4 mM IPTG.

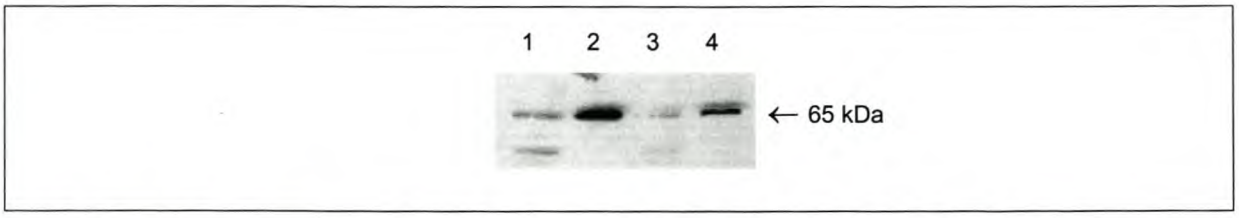


Fig. 4.5 A western blot analysis of the soluble and insoluble fractions of the proteins expressed from BL21 (DE3) pLysS bacterial cells transformed with the pET-WT or pET-Mut plasmids, induced with 0.1 mM IPTG and grown at 30°C. Lane 1, proteins from the soluble fraction of BL21 (DE3) pLysS (pET-WT) bacterial cells; Lane 2, proteins from the insoluble fraction of BL21 (DE3) pLysS (pET-WT) bacterial cells; Lane 3, proteins from the soluble fraction of BL21 (DE3) pLysS (pET-Mut) bacterial cells; Lane 4, proteins from the insoluble fraction of BL21 (DE3) pLysS (pET-Mut) bacterial cells.

4.3.3 PROTEIN PURIFICATION AND REFOLDING

Attempts at protein purification were only conducted on proteins expressed from bacteria induced with 0.4 mM IPTG and grown at 37°C. Insoluble inclusion body proteins were estimated to have a purity of approximately 90% (SDS-PAGE analysis), which is equivalent to that in other reports (Burgess, 1996, pET operator's manual), and contained 150 to 200 mg of protein per 500 ml of culture volume. Large aggregates of flocculent proteins were observed after dialysis with TEN50 buffer that did not contain 1 mM MgCl. When 1 mM MgCl was included in the dialysis buffer, the amount of flocculent protein was visibly reduced. Refolded proteins were concentrated and further purified by DEAE anion exchange chromatography.

4.3.4 DEAE ANION EXCHANGE CHROMATOGRAPHY

Hsp70h could not be distinguished by SDS-PAGE analysis in the protein profile obtained from the eluate from the DEAE sepharose matrix at the different salt concentrations (Fig. 4.6a). Western Blot analysis, however, was able to detect the WT-Hsp and Mut-Hsp proteins in the eluate between 16% and 22% TEN1000. Very little Hsp protein was detected in the 14% TEN1000 wash. The WT-Hsp and Mut-Hsp proteins were detected in the 16, 18, 20 and 22% TEN1000 eluate. The WT-Hsp and Mut-Hsp proteins were not detected in the 24% or the 26% TEN1000 eluates (Fig. 4.5b). The purity and concentration of the WT- and Mut-Hsp proteins was not sufficient to perform biochemical assays to determine the effect of the mutations on the ATPase activity.

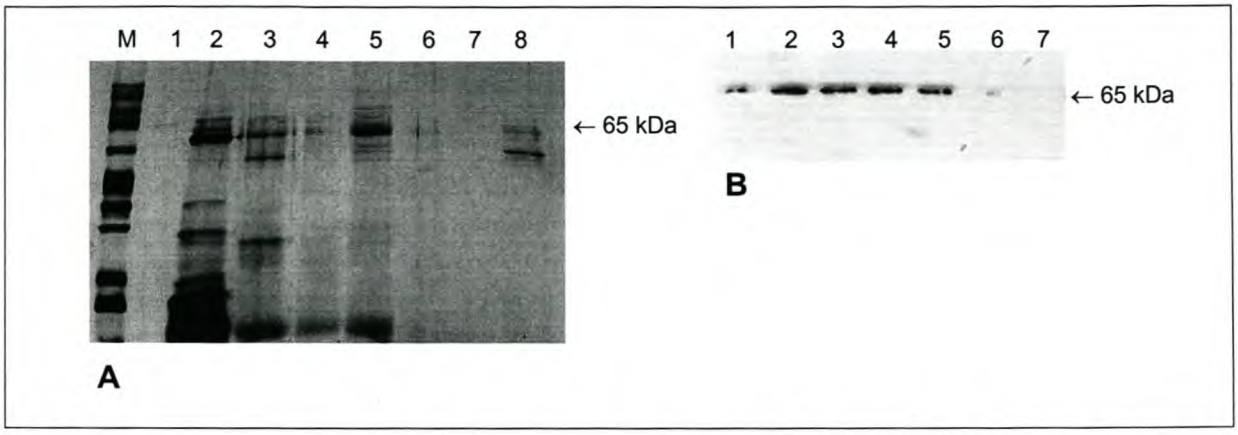


Fig. 4.6 Protein and western blot analysis of proteins eluted from the DEAE anion exchange chromatography from BL21 (DE3) pLysS (pET-Mut) bacterial cells, induced with 0.4 mM IPTG and grown at 37°C. Panel A) Silver stained SDS-PAGE gel. Lane M, Wide-range marker (Sigma); Lane 1, Hsp70h marker; Lane 2, proteins eluted from the 14% TEN1000 wash; Lane 3, proteins eluted from the 16% TEN1000 wash; Lane 4, proteins eluted from the 18% TEN1000 wash; Lane 5, proteins eluted from the 20% TEN1000 wash; Lane 6, proteins eluted from the 22% TEN1000 wash; Lane 7, proteins eluted from the 24% TEN1000 wash; Lane 8, proteins eluted from the 26% TEN 1000 wash. Panel B) western blot detected with the ECL detection system. Lane 1, proteins eluted from the 14% TEN1000 wash; Lane 2, proteins eluted from the 16% TEN1000 wash; Lane 3, proteins eluted from the 18% TEN1000 wash; Lane 4, proteins eluted from the 20% TEN1000 wash; Lane 5, proteins eluted from the 22% TEN1000 wash; Lane 6, proteins eluted from the 24% TEN1000 wash; Lane 7, proteins eluted from the 26% TEN 1000 wash.

4.4 DISCUSSION

The mutagenesis of the GLRaV-3 Stel isolate *hsp70h* gene was achieved by site-directed mutagenesis. Three mutagenic primers containing four point mutations were incorporated into the *hsp70h* gene in a single round of mutagenesis. These mutations have previously been shown to reduce the ATPase activity of Hsp70s. The Asp^{7} and Glu^{181} acidic amino acids of BYV have been shown to interfere with the movement and assembly of BYV (Peremyslov *et al.*, 1999). These amino acids correspond to the mutated Asp^{6} and Glu^{174} amino acids in GLRaV-3 Hsp70h.

Expression of the ΔHsp3 protein was achieved and the protein was purified for antibody production by gel elution. The antibody had a dilution factor of 1 in 20 000 when tested against the homologous purified protein. This C-terminal 20 kDa portion of the protein was selected for antibody production due to the lack of homology in this domain of the *Closteroviridae* and cellular Hsp70 proteins. In choosing a domain that had no homology to other related proteins, we reduced the chances of cross-contamination with cellular Hsp70s found in the expressing bacteria. The specificity of the antibody can be seen in the western blot results, which show that the antibody only reacted with a protein of approximately 65 kDa in cells that had been induced for protein expression or in the insoluble fraction and not with proteins from un-induced cells.

Expression of the WT- and Mut-Hsp proteins was achieved in BL21 (DE3) pLysS cells transformed with the corresponding pET-derived plasmid. Induced cells

produced a protein of approximately 65 kDa that was absent in uninduced cells. No significant difference was observed in the levels of proteins produced from the harvested cells. These proteins were expressed in inclusion bodies and therefore were located in the insoluble fraction. Attempts to increase the expression of proteins into the soluble fraction of the bacterial cells were unsuccessful. These attempts included reducing the concentration of IPTG for the induction of the T7 promoter and reducing the growth temperature of the induced cells. The Hsp proteins were detected in the soluble fraction of cells induced with a lower concentration of IPTG and grown at lower temperatures, but the levels of protein were lower by orders of magnitude when compared to the insoluble protein fraction. Further improvements in the expression could have been obtained by inducing the promoter with a lower concentration of IPTG, followed by overnight growth at 15 to 20°C, but this was not attempted.

The cloning strategy used for the expression of the WT- and Mut-Hsp proteins from the pET14b expression vector resulted in the removal of the solubility-enhancing His tag at the N-terminal end of the protein. The chosen cloning strategy did not require the re-PCR amplification of the genes and negated the difficulties associated with in-frame cloning in the limited multiple cloning site of the pET14b vector. Furthermore, the cloning strategy resulted in the ATG start codon of the *wt-* and *mut-hsp* genes being located in the optimal 8-nucleotide position downstream from the ribosome binding site. If, however, a different strategy was used for cloning the *wt-* and *mut-hsp* genes that maintained the 6 x His tag, soluble Hsp protein may have been isolated.

Protein solubilisation, refolding and anion exchange chromatography did not yield the pure protein required for biochemical tests. Sarkosyl was chosen as a solubilisation agent, as it can reduce the aggregation of proteins in the refolding process and is less harsh than the reduced environment created by urea and guanidine (Burgess, 1996). In addition, MgCl was included in the dialysis buffers to act as a co-factor and to stabilise the protein while it was undergoing refolding, as well as to improve the recovery of correctly folded proteins (Darby and Creighton, 1995). Neither of these additions appeared to have any effect on the amount of active soluble protein that was purified. Protein purity and protein concentration decreased significantly after refolding and DEAE anion exchange chromatography, as noted by SDS-PAGE analysis. The reason for this dramatic decrease in purity and the significant loss of protein is unknown. A possible explanation could be the poor refolding of proteins due to inefficient protein folding dynamics in the dialysis buffers. This incorrectly folded protein would have precipitated in the centrifugation clarification step following dialysis. Factors that affect the refolding of protein into the correct conformation are numerous. Not all of these factors were considered in our refolding experiments, but they should be taken into account for further work with protein purification of the WT-Hsp and Mut-Hsp proteins. These factors are the alkaline pH of the solution, the high molarity of the Tris buffer, the presence of

detergents (N-lauroylsarcosine) and polyethylene glycol 3500, metal ion ligands, redox systems and Cu^{2+} to catalyse air oxidation.

Numerous approaches can be employed to improve the refolding of insoluble proteins (Darby and Creighton, 1995). Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be determined empirically. In these experiments it was not possible to refold and purify sufficient quantities of the WT-Hsp or Mut-Hsp proteins for biochemical assays. Different expression strategies or systems could be used to obtain biochemically active proteins on which biochemical assays can be performed to test the effect of the mutations on the ATPase activity of the GLRaV-3 WT- and Mut-Hsp proteins. The alternate expression systems that could be used include the baculovirus or the *potato X potexvirus* plant expression systems. Both of these systems should overcome the problem of insoluble protein production, as the eukaryotic systems do not give rise to insoluble inclusion bodies. Ideally, the *potato X potexvirus* system could be used as the proteins would be expressed in the natural plant host. The significance of the negative result reported in this chapter will be discussed in Chapter 6.

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CHAPTER 5

RESEARCH RESULTS

A dysfunctional *grapevine leafroll-associated virus-3* Hsp70h protein confers resistance against the unrelated *potato X potexvirus* in transgenic tobacco

A DYSFUNCTIONAL *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3* Hsp70h PROTEIN CONFERS RESISTANCE AGAINST THE UNRELATED *POTATO X POTEXVIRUS* IN TRANSGENIC TOBACCO

5.1 INTRODUCTION

Leafroll is one of the three most important viral diseases of grapevine (Martin *et al.*, 2000) and has an effect on the quality and quantity of fruit (Goheen, 1988). Grapevine leafroll disease is a worldwide problem and it spreads in all or most grape-growing areas in which it is found. This spread is due to insect vector transmission by mealybugs and scale insects and the planting of virus-infected propagating material. Nine different members of the *Closteroviridae* are associated with leafroll disease. However, only *grapevine leafroll-associated virus-1* (GLRaV-1) and *grapevine leafroll-associated virus-3* (GLRaV-3) have been shown to be definitive aetiological agents of leafroll disease (Martelli, 1997).

Strategies for the control of leafroll disease are limited to vector control, plant sanitation and the supply of "virus-free" planting material. However, these approaches have not proven adequate to eliminate or control the disease, particularly in areas that have a high incidence of leafroll disease.

New molecular approaches based on the principle of pathogen-derived resistance (PDR) (Sanford and Johnson, 1985) have proven successful for the control of numerous viruses. Pathogen-derived resistance to plant viruses can be achieved with any coding or non-coding sequence of the virus. Pathogen-derived resistance operates on a number of different mechanisms, based on protein or RNA expression (Baulcombe, 1996). Protein-based mechanisms appear to interfere with a normal step in the virus infection cycle. RNA-based mechanisms appear to activate a natural plant defence mechanism that is related to post-transcriptional gene silencing. Post-transcriptional gene silencing depends on homology-pairing between the transgene-viral RNA or between two copies of the transgene mRNA, resulting in prevention of the accumulation of RNA that is homologous to the transgene (Grant, 1999). This process results in very high levels of resistance, however, the resistance is very specific to the targeted gene. The expression of RNA from the target gene can hybridise to viral RNA and form dsRNA. The dsRNA is digested into small interfering RNA (siRNA's) of 21 to 23 nucleotide lengths by the enzymatic cleavage of dsRNA by a "Dicer" RNase III. The siRNA's form a complex with the nuclease to form a RNA induced silencing complex (RISC). The RISC is activated by ATP dependent unwinding of the siRNA. The active RISC targets homologous transcripts and cleaves the mRNA or viral RNA.

Pathogen-derived resistance was first reported by Powell-Abel *et al.* (1986) for the *tobacco mosaic tobamovirus* (TMV) coat protein (CP). The CP is the most widely

used gene in PDR strategies and has been used on more than 30 different viruses (for reviews see Hackland *et al.*, 1994; Kavanagh and Spillane, 1995). Coat protein-mediated resistance (CPMR) offers good levels of resistance, but the resistance is specific to the homologous virus or closely related viruses. Replicase-mediated resistance (RepMR) also offers very good levels of resistance to the homologous virus, and this resistance is usually very specific against the homologous virus (Barbier *et al.*, 2000; Golemboski *et al.*, 1990; Thomas *et al.*, 2000). Movement protein-mediated resistance (MPMR) is usually protein based and can provide resistance against unrelated viruses (Kavanagh and Spillane, 1995).

An important phase of plant infection is the symplastic cell-to-cell movement of viruses via the plasmodesmata. The cell-to-cell movement from the site of the initial infection and the subsequent systemic infection of the plant is virus encoded. Some movement proteins (MPs) have been found to be interchangeable (Taliansky *et al.*, 1982). MPMR has important implications for crops that can be infected by multiple viruses, such as the grapevine. MPMR can offer broad-spectrum resistance to unrelated viruses if plants express a dysfunctional form of the MP (Beck *et al.*, 1994; Cooper *et al.*, 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.*, 1997; Tacke *et al.*, 1996). This dysfunctional form of the MP outcompetes the virus-encoded MP and therefore inhibits virus movement (Baulcombe, 1996; Seppänen *et al.*, 1997). The resistance to unrelated viruses is protein based. MPMR can be RNA based, although this form of resistance is only effective against the homologous virus (Tacke *et al.*, 1996). Plants transformed with and expressing the functional MP do not show any resistance to the virus and may have increased susceptibility to the infecting virus (Cooper *et al.*, 1995; Lapidot *et al.*, 1993; Ziegler-Graff *et al.*, 1991). The level of resistance obtained with MPMR can vary with the different viruses (Beck *et al.*, 1994; Cooper *et al.*, 1995). The resistance is caused by a delay in virus movement and is observed as a delay in the onset of symptoms (Cooper *et al.*, 1995; Lapidot *et al.*, 1993) and a reduction in the accumulation of the virus within the plant (Beck *et al.*, 1994; Cooper *et al.*, 1995; Tacke *et al.*, 1996). Improved knowledge of the MP and better targeting of the essential amino acids required for movement may result in increased levels of resistance (Lomonossoff, 1995).

The heat shock protein 70 kDa homolog (Hsp70h) is conserved amongst all members of the *Closteroviridae*. It has recently been shown to play an active role in the movement of *beet yellows closterovirus* (BYV) and can therefore be regarded as a definitive plant virus MP. Both the amino- and carboxy-terminal domains and functional ATPase activity are required for protein function with respect to virus movement (Peremyslov *et al.*, 1999). Four other proteins are also required for virus movement in BYV, viz. the small hydrophobic protein (p6), the 64 kDa protein (p64), the CP, and the divergent-CP (dCP) (Alzhanova *et al.,* 2000). Peng *et al.* (2001) also suggested that the BYV leader protease plays an important, but indirect, role in the cell-to-cell movement of the virus.

The Hsp70h protein has no structural similarities to other virus MPs, but does have some similar features. First, the protein is expressed early in infection (Hagiwara *et al.*, 1999); second, it is found to be associated with the plasmodesmata (Medina *et al.*, 1999); and third, it has an *in vitro* microtubule-binding ability (Karasev *et al.*, 1992). Two other types of MPs have been found to possess ATPase activity (Carrington *et al.,* 1998; Donald *et al.,* 1997). It has been speculated that elongated viruses may require extra energy for movement and cellular Hsp70s or MPs that have an ATPase activity therefore may play a greater role in virus movement than previously thought (Soellick *et* al., 2000). Peremyslov *et al.* (1999) speculated that ATP hydrolysis might be required to generate a force to facilitate viral translocation towards and through the plasmodesmata, a role similar to that of the cellular Hsp70s, which are thought to pull proteins across organellar membranes (Glick, 1995). The ATPase activity of the Hsp70h may provide the extra energy required for the translocation of the elongated viruses (Alzhanova *et al.*, 2001). It is unknown whether the Hsp70hs interact with viral nucleic acids or proteins, but there is evidence to suggest that the Hsp70h interacts with virions (Alzhanova *et al.*, 2001; Medina *et al.*, 1999; Napuli *et al.*, 2000; Tian *et al.*, 1999).

A model for the assembly and translocation of BYV has been proposed by Alzhanova *et al.* (2001). First, the virion body undergoes self-assembly of the RNA and the CP. This is followed by the formation of the virus tail, which is made up from the dCP and Hsp70h. The tail is attached to the virus body by the Hsp70h, a process which may be facilitated by ATP hydrolysis. The virion particle is then chaperoned to the plasmodesmata via an association of the integrated Hsp70h with the cell cytoskeleton (Karasev *et al.*, 1992) and is anchored in the plasmodesmata by an Hsp70h-derived localisation signal (Medina *et al.*, 1999). The translocation of the virion through the plasmodesmal channel may be facilitated by an Hsp70h-generated force, similar to the proposed cellular Hsp70 “trapping and pulling” translocation of proteins into the mitochondrion and endoplasmic reticulum (Pilon and Sheckman, 1999). Relocalisation of the Hsp70h to the plasmodesmata may trigger the disassembly of the tail. This may destabilise the virion body, resulting in a short region of RNA being exposed, which can interact with the ribosomes and start a new cycle of replication in the new cell. The Hsp70h is therefore not only required for BYV movement, but also for BYV assembly.

In this chapter, the cloning of the wild-type and mutated analogue of the GLRaV-3 *hsp70h* gene into a plant expression vector is reported. The Mut-Hsp contains four point mutations in conserved amino acids required for ATPase activity (Gaut and Hendershot, 1993; Wilbanks *et al.*, 1994). The WT-Hsp and Mut-Hsp constructs were transformed into *Nicotiana tabacum* cv. Petit Havana SR1 to test whether these constructs would confer resistance to an unrelated virus. Resistance to the homologous GLRaV-3 was not determined due to the inability of this virus to infect *N. tabacum* cv. Petit Havana SR1 or any other herbaceous host. The transformed plants were regenerated and characterised to determine transgene integration and

expression patterns. Finally, randomly selected plants were challenged with the unrelated *potato X potexvirus* (PVX) to determine whether resistance to an unrelated virus could be achieved in the transgenic plants containing the mutated Hsp70h (Mut-Hsp) construct. Virus challenge experiments with the homologous GLRaV-3, was not attempted due to the inability to infect GLRaV-3 into tobacco plants. Furthermore resistance to the homologous virus could be due to RNA-mediated resistance mechanisms which do not provide resistance to unrelated viruses. The resistance was observed in two plant lines. Both these plant lines contained a single copy of the transgene and both plant lines had high levels of mRNA transcript. The different integration patterns observed in these plant lines suggest that the resistance was due to the presence of the *mut-hsp* transgene and not due to the site of integration.

5.2 MATERIALS AND METHODS

5.2.1 VIRUS ISOLATES AND MAINTENANCE

Double-stranded RNA of GLRaV-3 was isolated from commercial Semillon grapevines grown in the Stellenbosch region of South Africa. The virus-infected material was collected in late summer and used immediately for dsRNA isolation according to the method of Rezaian and Krake (1987) as described in Chapter 3. An isolate of potato X potexvirus was supplied by Dr. Gerhard Pietersen (Agricultural Research Council – Roodeplaat, Pretoria) and was maintained in *Nicotiana benthamiana* and *N. tabacum* cv. Soulouk plants.

5.2.2 GENE CLONING AND PLANT TRANSFORMATION

Detailed descriptions of the PCR amplification and mutagenesis of the *hsp70h* gene can be found in Chapters 3 and 4. The wild type (*wt-hsp*) and mutated form of the Hsp genes (*mut-hsp*) were excised from the pAlter mutagenesis vector with *Eco*RI and cloned into the *Eco*RI site of the pART7 cloning vector (Gleave, 1992). The constructs were cut out of the cloning vector with *Not*I and cloned into the binary vector pART27 (Gleave, 1992). The resulting plasmids, pART-WT and pART-Mut, contained either the WT-Hsp or Mut-Hsp constructs driven by the constitutive CaMV35s promoter and octopine synthase (ocs3) terminator, and the neophosphotransferase (*nptII*) marker gene driven by the nopaline synthase (nos) gene promoter and terminator, located within the transfer-DNA (T-DNA) borders. The pART-WT and pART-Mut constructs were co-integrated into *Agrobacterium tumefaciens* (strain EHA 105) by tri-parental mating in the presence of helper plasmid (pRK2013). Transfer DNA was mobilised into *N. tabacum* cv. Petit Havana SR1 leaf discs from *Agrobacterium tumefaciens* according to the method of Horsch *et al.* (1985). Transformed shoots were regenerated on MS media (Murashige and Skoog, 1962) containing 30 g/l sucrose, 4 g/l Difco agar, 1 mg/l 6-benzyl-aminopurine (BAP), 120 μg/l kanamycin and 400 μg/l cefotaxim (Claforan™). The shoots that developed

were removed and placed on MS media containing 30 g/l sucrose, 4 g/l Difco agar, 0.5 mg/l α naphthalene acetic acid (NAA), 120 μg/l Km and 400 μg/l cefotaxim. Rooted plants were multiplied and hardened off in a growth room (24 ± 5°C, 16 h light and 8 h dark). The R_0 transformed plants were self-pollinated and the R_1 seeds were collected. The R_1 seeds were geminated and grown in soil for virus challenge experiments.

5.2.3 GENETIC EVALUATION OF TRANSFORMED CELL LINES

Tobacco genomic DNA was isolated from hardened-off plants, essentially according to the method of McGarvey and Kape (1991), with the use of N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) and phenol chloroform extraction. Ten μg of genomic DNA was digested with *Bam*HI, separated on a 0.8% agarose gel and transferred to Hybond N+ nylon membrane (Amersham) by capillary transfer (Sambrook *et al.*, 1989). Southern hybridisation was performed as per the manufacturer's instructions (Roche Biochemicals), using the *wt-hsp70* gene PCR-labelled with DIG-dUTP as a probe.

Total RNA was isolated from hardened-off plants with the TRIZOL reagent (Gibco-BRL) as per the manufacturer's instructions. Equal aliquots of RNA were denatured and separated on a 1.2% formaldehyde agarose gel (Sambrook *et al.*, 1989) and transferred to Hybond N+ nylon membrane (Amersham) by capillary transfer. Northern hybridisation was performed as per the manufacturer's instructions (Roche Biochemicals) with a *wt-hsp* DNA probe PCR-labelled with DIG-dUTP. The membranes were stripped and reprobed with a 16S RNA DIG-dUTP-labelled probe to act as a standard (Fig 5.2b and d).

Total cell protein was isolated from selected plant lines, separated on a 10% SDS-PAGE gel (Laemmli, 1970) and subjected to western blot analysis. For western blot analysis, gels were electrophoretically transferred to Hybond C-extra nylon membranes (Amersham Pharmacia Biotech) as described by Towbin *et al.* (1979). The antibody raised against the expressed C-terminal domain of the GLRaV-3 Hsp70h was used to detect antigen (Chapter 4). The antibody-antigen complex was detected with the ECL detection kit (Amersham Pharmacia Biotech).

5.2.4 VIRUS INOCULATION AND DETECTION

Potato X potexvirus was purified from systemically infected *N. tabacum* cv. Soulouk leaves. Purified preparations of PVX were diluted to 5 and 20 μg in 100 μl of 0.01 M phosphate buffer (pH 7.6) and mechanically inoculated onto the leaf tips of the lowest fully expanded carborundum-dusted leaf of transgenic R_1 self-progeny *N. tabacum* cv. Petit Havana SR1 plants. Transgenic plants were inoculated when the plants had four fully expanded leaves and developing leaflets (approximately 10 weeks after sowing). Plant lines C2, M1, M5, M6, W1, W12 and W19 were inoculated with 20 μg of PVX. Plant lines C2, M5 and M6 were inoculated with 5 μg of PVX. In the confirmatory experiments, plant lines C2 and M10 were inoculated with 5 μg of PVX.

Samples of the three leaves situated directly above the inoculated leaf were collected at 9, 11, 13 and 15 days post inoculation (d.p.i.) from plants infected with 20 μg of PVX. Samples were processed separately. A sample of the uninoculated portion of the inoculated leaf was collected at 15 d.p.i from plants infected with 20 μg of PVX to determine whether the infection had been successful. Samples from two systemically infected apical leaves were collected at 43 d.p.i. and were processed together. Samples from three systemically infected leaves were collected at 20 and 30 d.p.i. from transgenic plants infected with 5 μg of PVX and processed. In total, eight M5 and two M10 plants were inoculated in independent experiments with PVX. All samples were processed according to La Notte *et al.* (1997). Essentially, the leaf discs were ground in liquid nitrogen and resuspended in buffer 1 (0.01 M carbonate buffer, pH 9.6, 0.005% Tween 20, 2% PVP40, 0.2% BSA, 1% sodium metabisulphite). Two μl aliquots were added to GES buffer (100 mM glycine, pH 9, 1 mM EDTA, 50 mM NaCl, 0.5% TritonX100) and heated at 95°C for 10 min. The solution was flash cooled on ice and 2 μl was added to the RT-PCR tube.

Reverse transcription-PCR of an internal portion of the coat protein (CP) gene of PVX with oligonucleotide primers PVX-F 5'-CAACTACTGCCACAGCTTCAGGA-3' and PVX-R 5'-GGCAGCATTCATTTCAGCTTCAGAC-3' was used to determine whether PVX had replicated and systemically infected the plants. The PCR reactions were carried out in a standard 25 μl one step RT-PCR reaction containing 1 u AMV reverse transcriptase (Promega), 1 u BioTaq (Bioline), 20 μM PVX-F and 20 μM PVX-R oligonucleotide primers and 1 mM of each dNTP. The amplification reaction included an initial step of 42°C for 45 min to convert the RNA into cDNA, followed by an initial denaturation step of 94°C for 4 min. Thirty cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec were followed by a final elongation step of 72°C for 5 min. Five μl aliquots of the RT-PCR reactions were run on a standard 1% TAE agarose gel and viewed under UV light with ethidium bromide staining (Sambrook *et al.*, 1989).

5.3 RESULTS

5.3.1 TRANSFORMATION OF *N. TABACUM* CV. PETIT HAVANA SR1 WITH *WT-HSP* AND *MUT-HSP* CODING SEQUENCES

The complete coding sequence of a wild type grapevine leafroll-associated virus-3 *hsp70h* gene and a mutated homologue of the *hsp70h* were cloned into the pART27 binary vector. These constructs were used to transform *N. tabacum* cv. Petit Havana SR1 via *A. tumefaciens*. Eighteen Km^r plantlets were obtained for the Mut-Hsp construct (designated M plant lines) and 22 Km^r plantlets were obtained for the WT-Hsp construct (designated W plant lines). Genomic DNA was isolated from all 40 plantlets and two negative controls (designated C plant lines). These DNA isolations were subjected to Southern hybridisation with a DIG-labelled full-length WT-Hsp

probe. Of the 18 Mut-Hsp Km^r lines, five plant lines did not appear to contain a copy of the transgene. The 13 remaining plant lines contained between one and four copies of the transgene (Table 5.1, Fig. 5.1a). Of the 22 WT-Hsp Km^r plant lines, six plant lines did not appear to contain a copy of the transgene, while the remaining 16 plant lines contained between one and six copies of the transgene (Table 5.1, Fig. 5.1b). The two control plant lines did not have any copies of the transgene and no mRNA transcript was detected. Transgene expression levels were determined by Northern hybridisation and indicated that the levels of transcript accumulation varied between the different plant lines (Table 5.1, Fig. 5.2a and b). The mRNA transcript levels were standardised with a rRNA probe (results not shown). Hybridisation analysis indicated that *mut-hsp* mRNA was expressed in 12 plant lines and that *wt-hsp* mRNA was expressed in 15 plant lines. The plants were hardened off for seed collection and self-pollinated. Five WT-Hsp plant lines were sterile and no seeds were collected from these plants (Table 5.1).

Western blot analysis was inconclusive. Proteins of the expected size (65 kDa) were detected in all plant lines, including the control plant line (Fig 5.3). This indicates that the antibody could detect non-specific proteins in plants.

Table 5.1 The copy number of the Mut-Hsp or the WT-Hsp transgenes in the different transgenic plant lines as determined by Southern hybridisation and the correlation with transcript expression levels as determined by Northern hybridisation. Two non-transformed plants lines were included as controls.

Plant line	Copy number	Transcript expression	Plant line	Copy number	Transcript expression
C1	0	-	W1	0	-
C2	0	-	W2	0	-
			W3	0	-
M1	0	-	W4	0	-
M2	0	-	W5	>3	++
M3	2	-	W6	3	+
M4	0	-	W7	3	+
M5	1	++	W8*	5	+
M6	4	+++	W9	4	+
M7	0	-	W10	1	-
M8	>4	+	W11*	0	-
M9	>4	++	W12	>6	+
M10	1	++	W13	>2	+
M11	2	++	W14	3	++
M12	2	++	W15	2	+
M13	1	+++	W16*	3	+
M14	0	-	W17	>3	++
M15	1	+	W18*	3	+
M16	1	+	W19	3	+
M17	2	+++	W20	0	-
M18	2	+++	W21	1	++
			W22*	1	++

* Sterile plants
\- No detectable expression
\+ Low expression
++ High expression
+++ Very high expression

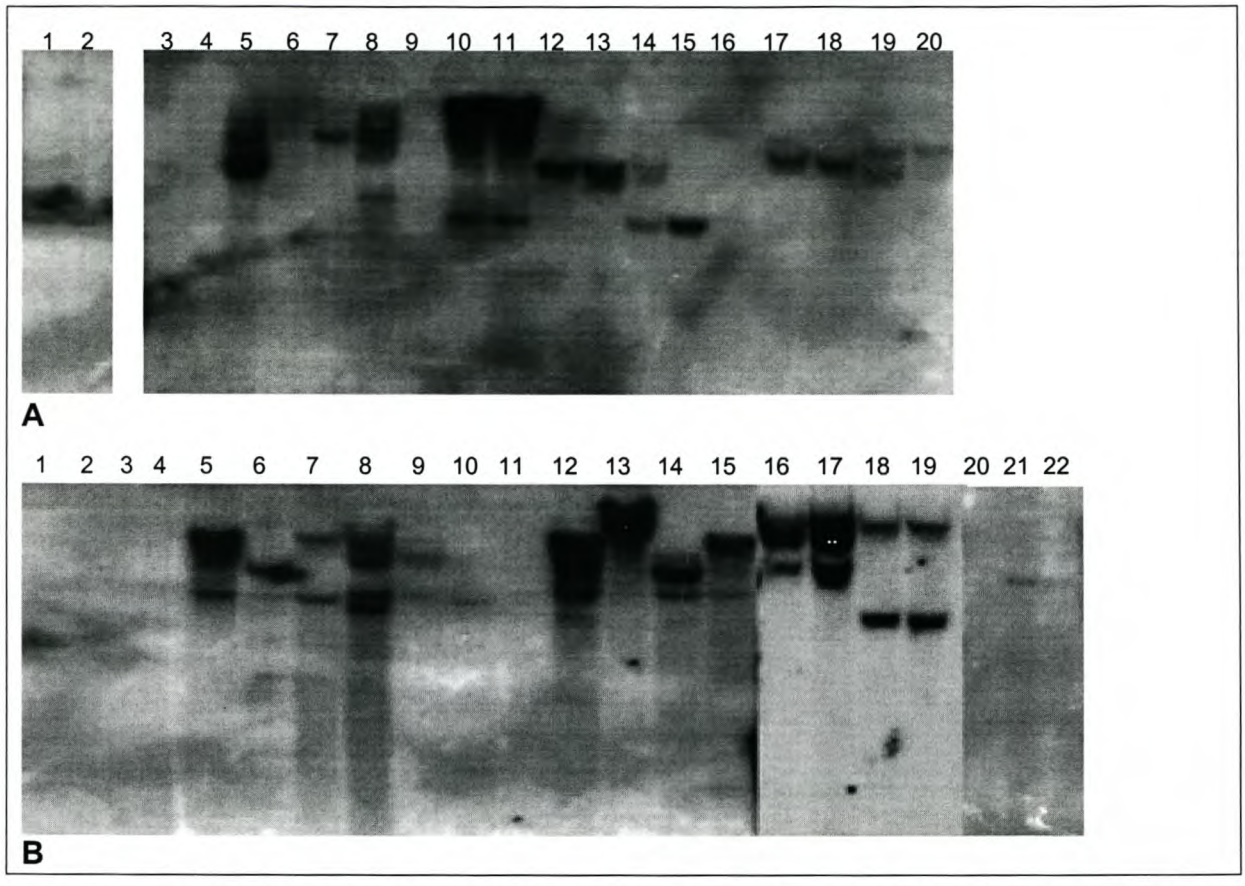

Fig. 5.1 Autoradiographs of Southern hybridisations showing the transgene copy number. Panel A) Southern hybridisation of the control and Mut-Hsp plant lines. Lanes 1- 2, DNA from control plant C1-C2; Lanes 3-20, DNA from Mut-Hsp plant lines M1-M18. Panel B) Southern hybridisation of the WT-Hsp plant lines. Lanes 1-22, DNA from WT-Hsp plant lines W1-W22. Membranes were hybridised with a full-length WT-Hsp DNA probe PCR labelled with DIG-dUTP

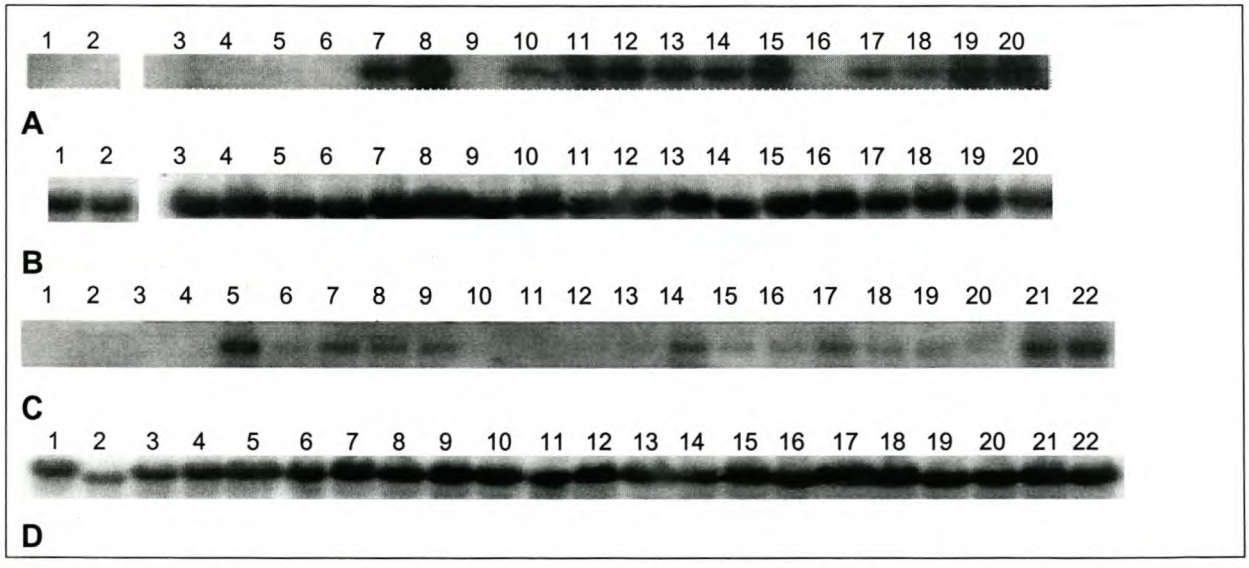

Fig. 5.2 Autoradiographs of Northern hybridisations showing the transgene expression levels. Panel A and B) Northern hybridisation of the control and the Mut-Hsp plant lines. Lanes 1- 2, RNA from control plant C1-C2; Lanes 3-20, DNA from Mut-Hsp plant lines M1-M18. Panel C and D) Northern hybridisation of the WT-Hsp plant lines. Lanes 1-22, RNA from WT-Hsp plant lines W1-W22. Membranes A and C were hybridised with a full-length WT-Hsp DNA probe PCR labelled with DIG-dUTP. Membranes B and D were hybridised with a 16s rRNA probe.

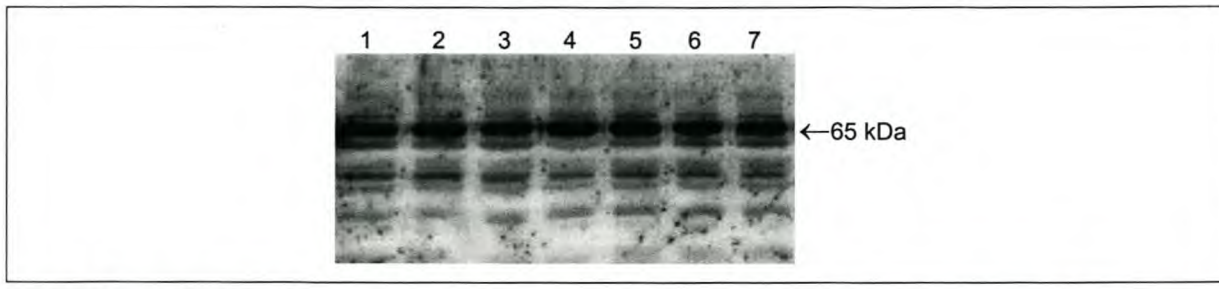

Fig 5.3 Western blot analysis of transgenic plants. Total cell protein was isolated and run on a 10% SDS-PAGE gel. Proteins were detected with the GLRaV-3 Hsp C-terminal protein domain. Lane1, plant line C2; Lane 2, plant line M1; Lane 3, plant line M5; Lane 4, plant line M6; Lane 5, plant line W1; Lane 6, plant line W12; Lane 7, plant line W19.

5.3.2 ACCUMULATION OF PVX

Potato X potexvirus accumulated in all plant lines challenged with 20 μg of PVX, except in plant line M5. PVX was detected in plant lines M1, M6, W1, W12 and W19 at 9 d.p.i. *Potato X potexvirus* was only detected in the control plant line at 11 d.p.i. *Potato X potexvirus* appeared to have moved into all the sampled leaves of plant lines M1 and W12 by 11 d.p.i., into the sampled leaves of plant lines M6 and W1 by 13 d.p.i and into the sampled leaves of plant line W19 by 15 d.p.i. The control plant did not appear to have accumulated PVX in all sampled leaves by 15 d.p.i. and only the two uppermost sampled leaves had accumulated PVX by 15 d.p.i. Plant line M5 did not appear to have any detectable PVX accumulation in any of the three sampled leaves at 9, 11, 13 or 15 d.p.i. (Fig. 5.4a). A very weak signal was detected in the apical sample leaf of plant line M5 at 11 d.p.i. This signal was not present at 13 and 15 d.p.i. and is presumed to be a contaminant acquired in the processing of the samples.

Apical leaves from the seven plant lines challenged with 20 μg of PVX were collected at 43 d.p.i. RT-PCR amplification indicated that all the plant lines were systemically infected with PVX, except plant line M5 (Fig. 5.4b). Uninoculated portions of the inoculated leaves of plants infected with 20 μg of PVX were collected at 15 d.p.i. and subjected to RT-PCR amplification. All samples tested positive for PVX infection, indicating that the virus inoculation procedure was successful in all plants (Fig. 5.4c).

Plant lines C2, M5 and M6 were inoculated with 5 μg of PVX. Systemically infected apical leaf samples were collected at 20 and 30 d.p.i. *Potato X potexvirus* accumulated in the apical leaves of plant lines C2 and M6, but did not accumulate in the apical leaves of plant line M5 at 20 and 30 d.p.i. (Fig. 5.5). Plant lines C2 and M10 were inoculated with 5 μg of PVX and virus accumulation was determined in the uninoculated portions of the inoculated leaf at 10 d.p.i., and in the systemic apical leaves at 20 and 30 d.p.i. *Potato X potexvirus* accumulated in the infected leaf of C2 and M10 plants, and in the systemically infected apical leaves of C2 at 20 and 30 d.p.i. *Potato X potexvirus* was not detected in the systemically infected apical leaves of plant line M10 at 20 and 30 d.p.i. (Fig. 5.6).

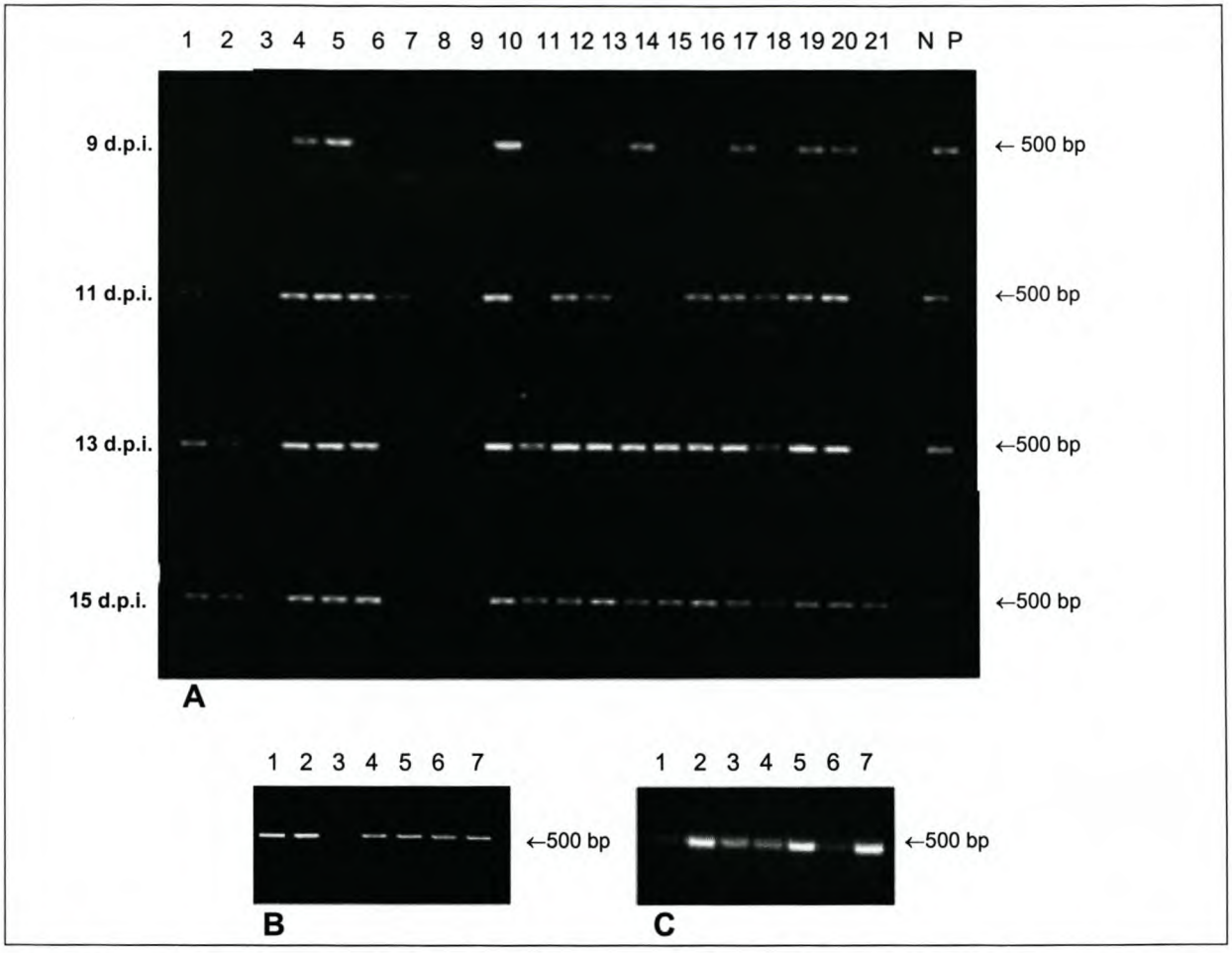

Fig. 5.4 An agarose gel showing the PVX accumulation in tobacco plants challenged with 20 μg of PVX as assayed by RT-PCR. Panel A) Leaf samples of the three leaves just above the inoculated leaf were collected at 9, 11, 13, and 15 d.p.i. One control plant, three Mut-HSP and three WT-HSP plants were tested for virus accumulation. C2 leaf samples are found in lanes 1-3, M1 leaf samples are found in lanes 4-6, M5 leaf samples are found in lanes 7-9, M6 leaf samples are found in lanes 10-12, W1 leaf samples are found in lanes 13-15, W12 leaf samples are found in lanes 16-18 and W19 leaf samples are found in lanes 19-21. The lower number leaf in each series represents the uppermost leaf sample and the higher numbers are found consecutively lower down the plant closer to the inoculated leaf. A negative (N) and a positive (P) control were included. Panel B) RT-PCR amplification of apical leaf samples of the infected plants at 43 d.p.i.; Lane 1, plant line C2; Lane 2, plant line M1; Lane 3, plant line M5; Lane 4, plant line M6; Lane 5, plant line W1; Lane 6, plant line W12; Lane 7, plant line W19. Panel C) RT-PCR amplification of uninoculated portions of the inoculated leaf collected at 15 d.p.i. to confirm virus infection; Lane 1, plant line C2; Lane 2, plant line M1; Lane 3, plant line M5; Lane 4, plant line M6; Lane 5, plant line W1; Lane 6, plant line W12; Lane 7, plant line W19.

Fig. 5.5 An agarose gel showing the PVX accumulation in tobacco plants challenged with 5 μg of PVX as assayed by RT-PCR. Panel A) Systemically infected apical leaves of plant lines C2, M5 and M6 collected at 20 d.p.i. Panel B) Systemically infected apical leaves of plant lines C2, M5 and M6 collected at 30 d.p.i. Lane 1, plant line C2 collected at 20 d.p.i.; Lane 2, plant line M5 collected at 20 d.p.i.; Lane 3, plant line M6 collected at 20 d.p.i.; Lane 4, plant line C2 collected at 30 d.p.i.; Lane 5, plant line M5 collected at 30 d.p.i.; Lane 6, plant line M6 collected at 30 d.p.i.

Fig. 5.6 An agarose gel showing the PVX accumulation in tobacco plants challenged with 5 μg of PVX as assayed by RT-PCR. Lane 1, Uninoculated portion of the C2 inoculated leaf sampled at 10 d.p.i. Lane 2, Systemically infected leaves of plant line C2 collected at 20 d.p.i. Lane 3, Systemically infected leaves of plant line C2 collected at 30 d.p.i. Lane 4, Negative control, uninoculated C2 leaves. Lane 5, Uninoculated portion of the M10 inoculated leaf sampled at 10 d.p.i. Lane 6, Systemically infected leaves of plant line M10 collected at 20 d.p.i. Lane 7, Systemically infected leaves of plant line M10 collected at 30 d.p.i.

5.4 DISCUSSION

The tobacco cultivar Petit Havana SR1 was transformed with the *wt-hsp* and *mut-hsp* gene constructs. Twenty-two plant lines were obtained from the WT-Hsp construct and 18 plant lines were obtained from the Mut-Hsp construct. All 40 of these plant lines appeared to have no somaclonal variation. These plants grew and appeared normal in all respects.

Southern hybridisation analysis of the transgenic plant lines indicated that six of the 22 WT-Hsp plant lines and five of the 18 Mut-Hsp plant lines did not contain a stably integrated copy of the transgene. Blake *et al.* (1991), Monier *et al.* (2000) and Valkonen *et al.* (1995) reported similar results for plants that appeared to contain the *nptII* gene and exhibit Km^r but did not contain a second gene that is also found within the same T-DNA. No significant differences were noted in the copy numbers of the WT-Hsp and Mut-Hsp plant lines, with all plants containing less than six copies of the respective transgenes. It is possible that plant lines M8, M9, W5, W12, W13 and W17 contain more transgene copies than indicated in table 5.1, as the high molecular weight DNA hybridises with greater intensity.

From the Southern blot analysis, it may be possible that plant lines M10 and M11 may be clones of each other, plant lines M15 and M16 may be clones of each other and plant lines W17 and W18 may be clones of each other and are therefore, not independent transformants. The transgene integration patterns of these lines look similar in the Southern blot analysis and may, therefore, be clones of each other. Although plant lines M8 and M9 also have a similar integration pattern, the mRNA transcript levels observed in the Northern blot are different. To confirm whether these plant lines are clones, or are independent transformants, DNA isolated from these plant lines can be digested with different restriction enzymes and the Southern blot banding patterns can be compared. If similar banding patterns are again observed, we will be able to conclude that the plant lines are indeed clonal variants.

Transcript expression levels of the *mut-hsp* transgene appeared to be higher than the transcript expression levels obtained by the *wt-hsp* transgene. Transgene expression levels did not correspond to the copy number of the transgenes, as seen with plant lines M5, M10, M13, M15 and M16, which all appear to contain a single

gene copy but have mRNA transcript levels varying from low to high. The variation in gene expression could be due to the site of insertion of the transgene within the plant genome. Twelve plants had detectable levels of the *mut-hsp* mRNA and 15 plants had detectable levels of the *wt-hsp* mRNA. Western blot results were inconclusive and it was not possible to determine whether protein was expressed in the transgenic plants due to the non-specific nature of the antibody when tested against proteins isolated from plants.

Potato X potexvirus was selected as the challenge virus for *in planta* tests of the F_1 generation of plants. This virus is distinct from and unrelated to GLRaV-3. It can systemically infect *N. tabacum* cv. Petit Havana SR1 and is mechanically transmissible. RT-PCR was chosen as the technique to detect the presence or absence of PVX in inoculated plants due to the sensitivity and specificity of the RT-PCR detection assay. All plant lines were infected with PVX, as shown by the RT-PCR amplification results of the uninoculated portions of the inoculated leaf sampled at 15 d.p.i. The infected control plant had detectable levels of PVX at 11 d.p.i. Plant lines M1 and W1 did not contain any copies of the respective transgenes and can therefore be regarded as "transformed" control plants containing the *nptII* gene. At 15 d.p.i., PVX was not detected in any of the three sampled leaves of plant line M5, even though the inoculated leaf had detectable levels of PVX at 15 d.p.i. The three remaining transgenic plants, M6, W12 and W19 containing transgene copies accumulated PVX. We expected plant lines W12 and W19 to accumulate PVX. These plants containing the functional transgene would not be able to interfere in the movement of viruses (Cooper *et al.*, 1995; Lapidot *et al.*, 1993; Ziegler-Graf *et al.*, 1991). The accumulation of PVX in plant line M6 was, however not expected. The M6 plant line has four copies of the transgene and has very high expression levels of the *mut-hsp* transgene, yet this plant line showed no detectable resistance to PVX. This result concurs with a finding by Seppänen *et al.* (1997), who reported that there was no correlation between virus resistance and mRNA transcript levels. We deduce that the *mut-hsp* transgene in plant line M6 may be subjected to post-translational silencing, or the protein was not expressed due to the site of integration of the transgenes in the plant genome (Monier *et al.*, 2000; Neves-Borges *et al.*, 2001).

The accumulation of PVX in plant lines C2, M1, M6, W1, W12 and W17 was confirmed by PVX CP accumulation. The rate of virus accumulation was not quantified as RT-PCR tested only for the presence or absence of PVX and amplification was not quantified. Plant lines C2 and W12 had low band intensities for the amplified CP of PVX in the uninoculated portions of the inoculated leaf sampled at 15 d.p.i. This could be due to inefficient inoculation or to the low levels of surviving virus in the older senescing inoculated leaves. The efficiency of infection is, therefore inconclusive. To overcome the apparent inefficiency of inoculation observed in these experiments, and to determine whether plant line M5 had a delay in virus accumulation or showed absolute resistance, systemically infected apical leaves were sampled again at 43 d.p.i. Plant line M5 had no detectable levels of PVX

accumulation, whereas the control plant, the two "transformed" control plants and the three plants containing the *wt-hsp* or *mut-hsp* transgenes appeared to have accumulated PVX to similar levels. This indicates that all of the plants were successfully inoculated with the challenging PVX and that all of these plants were systemically infected by the virus. In an independent test to confirm the resistance of plant line M5, three plant lines, C2, M5 and M6, were challenged with 5 μg of PVX. Only plant line M5 showed any form of resistance to the PVX infection in the systemic portions of the inoculated plants at 20 and 30 d.p.i. These virus accumulation results suggest that plant line M5 shows high levels of resistance to the unrelated PVX. To confirm that the resistance observed in plant line M5 against PVX infection was due to the incorporation and expression of the *mut-hsp* transgene, and not an aberrant result due to transgene incorporation, a second plant line containing a single transgene copy with a different integration pattern, as observed with Southern hybridisation, and with similar mRNA transcript levels was subjected to virus challenge with 5 μg of PVX. These M10 plants showed the same type of systemic resistance observed in the M5 plant line. Plant lines M5 and M10, therefore appeared to have complete resistance to PVX infection, as opposed to a delay in virus accumulation. Cooper *et al.* (1995) found that the dysfunctional MP from TMV provided systemic resistance to the unrelated viruses, but had no effect on the cell-to-cell movement in the inoculated leaf. These results are similar to the results obtained in this work, according to which PVX was able to infect the inoculated leaf but did not systemically infect the plant. Due to the complicated nature of plant virus movement and the lack of understanding concerning the long distance transport of viruses through the vascular tissue, we are unable to explain why the *mut-hsp* transgene is able to affect the long distance transport of PVX, but does not appear to affect the cell-to-cell movement of the virus, particularly in light of the role that the Hsp70h plays in cell-to-cell movement of *beet yellows virus* (Alzhanova, *et al.*, 2001).

Due to the high levels of *mut-hsp* mRNA expression obtained in plant lines M5 and M10, it can be deduced that the virus resistance in these plant lines are most likely protein mediated. RNA-mediated resistance is usually associated with low levels or no detectable levels of transgene expression (Moreno *et al.*, 1998; Rovere *et al.*, 2002; Sijen *et al.,* 1996; Smith *et al.*, 1994). Further support for a protein-mediated resistance mechanism comes from the highly specific nature of RNA-mediated resistance (Grant, 1999). No such sequence homology exists between the GLRaV-3 *hsp70h* and the PVX genome. However, until we are able to obtain definitive western blot data to show the presence of the Mut-Hsp70h protein in plant lines M5 and M10 and the absence of the protein in plant lines C2 and M6, we are unable to definitively conclude that the resistance is protein-based.

The results reported in this chapter are similar to those reported by other researches who found that plants containing a dysfunctional MP from a virus showed resistance against unrelated viruses (Beck *et al.*, 1994; Cooper *et al.*, 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.*, 1997; Tacke *et al.*, 1996).

Furthermore it has been shown that only a few of the transgenic plant lines yielded any form of resistance to homologous or heterologous viruses (Barbier *et al.*, 2000; Beck *et al.*, 1994; Krastanova *et al.*, 2000; Neves-Borges *et al.*, 2001), a result noted in these experiments. It is concluded that the tobacco plants containing the dysfunctional form of the GLRaV-3 *hsp70h* gene containing mutations that are proposed to reduce ATPase activity, are resistant against infection by the unrelated PVX. This result has been confirmed, as two plant lines containing a single copy of the *mut-hsp* transgene were resistant to PVX infection. These two plants have different transgene integration patterns, therefore it can be concluded that the resistance observed in these plants is due to the presence and expression of the *mut-hsp* transgene and not due to site of integration. It remains to be seen whether all plant lines containing a single copy of the *mut-hsp* transgene show this same resistance. It is also necessary to test tobacco plant lines M5 and M10 against other virus isolates in order to determine whether the *mut-hsp* transgene can provide resistance to different viruses and thus provide broad-spectrum resistance. The *mut-hsp* transgene also needs to be transformed into grapevine for resistance testing against the homologous GLRaV-3 and other known grapevine leafroll-associated viruses. Due to the host specificity of the grapevine-infecting members of the *Closteroviridae*, these tests need to be performed in grapevine plants. This work can now be attempted as we have proved that a dysfunctional form of the GLRaV-3 *hsp70h* can provide resistance against infection by an unrelated virus.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

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The variety of viruses associated with grapevine leafroll disease, have been well documented. However, the taxonomic classification of these viruses has not been determined, due to the difficulties of isolating pure and sufficient quantities of virus. A phylogenetic analysis, incorporating three South African *grapevine leafroll-associated virus-3* (GLRaV-3) isolates and the NY-1 isolate from the United States of America (Ling *et al.*, 1998) was undertaken. Our results showed that GLRaV-3 contains heterogeneity within the heat shock protein 70 kDa homolog (Hsp70h). This result is to be expected for a ssRNA virus. The four GLRaV-3 isolates used for phylogenetic analysis form two distinct groupings, with greater than 98% homology within each grouping and 94% homology between each of these two groupings. Furthermore, the three South African virus isolates shared 15 common mutations from the NY-1 isolate. This phylogenetic analysis confirmed the great diversity of the grapevine infecting members of the *Closteroviridae*, which are located within the *closterovirus* and *ampelovirus* genera. The GLRaV-7 isolate may also be reclassified as a member of the crinivirus genus, or in an as yet unidentified genus. However, the reclassification of GLRaV-7 needs further taxonomic information based on either the genome sequence, or the vector transmissibility of the virus. The phylogenetic analysis obtained in this study confers with the current taxonomy of the *Closteroviridae*. We propose that new *Closteroviridae* isolates can be tentatively classified into the appropriate genus by observing the phylogenetic analysis based on the *Hsp70h* gene.

The primary aim of this study was to prove the concept that a dysfunctional form of the GLRaV-3 movement protein (MP), the Hsp70h, could be used in a pathogen-derived resistance strategy to confer resistance against a distinct unrelated virus. Within the last two to three years, other researchers have shown the Hsp70h to be one of six proteins required for movement of *beet yellows closterovirus* (BYV). The p6, p64, CP, dCP and leader protease are also required for movement (Alzhanova *et al.*, 2001; Peng *et al.,* 2001), but play an indirect role. Furthermore, a hypothesis has been proposed about how the Hsp70h is involved in virus assembly and movement within the cell and translocation to adjacent cells (Alzhanova *et al.*, 2001). Mutations in two conserved acidic amino acids, Asp^{7} and Glu^{181}, which are located in the ATPase domain of the BYV Hsp70h, have been shown to abolish the movement of BYV in plants (Peremyslov *et al.*, 1999). To date, these mutated Hsp70hs have not been used in a pathogen-derived resistance strategy, but have only been used to elucidate a model for the movement of BYV (Alzhanova *et al.*, 2001). In this project, evidence is presented indicating that plants expressing a mutated form of the GLRaV-3 Hsp70h containing four point mutations, which were previously shown to be required for ATPase activity in cellular Hsp70s (Gaut and Hendershot, 1993;

Wilbanks *et al.*, 1994), are able to confer resistance against infection of the unrelated *potato X potexvirus* (PVX).

In order to stay within the aim and time frame of this study, it was decided to select and challenge only a few transgenic plant lines to prove the movement protein-mediated resistance concept with the GLRaV-3 dysfunctional-Hsp70h. This was decided due to the logistical difficulties and costs of RT-PCR amplification and time frame constraints. Three plant lines containing either the *wt-hsp* or *mut-hsp* transgene were randomly selected for virus challenge experiments. These plants were challenged with PVX, which can systemically infect *N. tabacum* cv. Petit Havana SR1 and has no taxonomic relationship to GLRaV-3. A fourth plant line containing the *mut-hsp* transgene was challenged with PVX to confirm whether the observed resistance was due to the presence of the transgene, or due to an integration event which could eliminate an unknown gene that is essential for systemic plant virus movement.

Original leaf sampling was designed to detect a delay in virus movement, as has previously been observed with movement protein-mediated resistance (Beck *et al.*, 1994; Cooper *et al.*, 1995; Lapidot *et al.*, 1993). However, plant line M5 did not appear to have delayed virus movement, but rather appeared to have total immunity to the challenging virus. Therefore, in follow-up experiments to confirm the resistance status of plant line M5, systemically infected apical leaves were sampled for virus accumulation at 20 and 30 days post inoculation (d.p.i.). The results obtained were as expected and eight M5 plants appeared to have complete immunity to PVX infection. To confirm that the observed resistance was due to expression of the *mut-hsp* transgene a second plant line, M10, with a single transgene copy and high mRNA transcript levels, was selected for PVX challenge experiments. The M10 plants were challenged with 5 μg of PVX. The accumulation of PVX was determined in the systemically infected apical leaves. The same type of systemic resistance, observed in the M5 plant line, was observed in two M10 plants. This confirmed that the observed resistance was due to the presence of the *mut-hsp* transgene, as opposed to insertion of foreign DNA into an essential plant gene required by viruses for long distance transport. The resistance was observed at two different levels of virus infection. Although virus challenge was with relatively low levels of virus, viz. 5 μg and 20 μg of PVX, resistance was observed at both these levels. Movement protein-mediated resistance can be overcome at high levels of virus infection (Cooper *et al.,* 1995; Seppänen *et al.*, 1997). With respect to grapevine leafroll disease, the virus levels are thought to be very low, as noted with virus titres in infected plants. The levels of virus transmitted by the mealybug and scale insect vectors are unknown, but are assumed to be low, below the levels tested in these experiments. Therefore, resistance observed at these virus levels should be sufficient to control the grapevine leafroll-associated viruses.

It was not possible to establish whether the point mutations incorporated into the GLRaV-3 Hsp70h did indeed have an effect on the ATPase activity of the mutated

protein. It is believed that the incorporated mutations did reduce the ATPase activity of the GLRaV-3 Hsp70h protein for two reasons: first, the mutated amino acids are highly conserved amongst members of the Hsp70 family and mutations in these amino acids have been shown to reduce ATPase activity (Gaut and Hendershot, 1994; Wilbanks *et al.*, 1993). Second, BYV requires the presence of ATPase activity for virus movement (Peremyslov *et al.*, 1999).

Active forms of the wild type and mutated proteins could not be purified in sufficient quantities for biochemical assays of ATPase activity. This result is of less significance in terms of the project, due to the virus resistance obtained in plant lines M5 and M10. It is believed that the mutations incorporated into the GLRaV-3 Hsp70h resulted in a decrease in ATPase activity. The level of decrease in ATPase activity is speculated to be very significant. Mutations in the heat shock cognate protein 70 kDa (Hsc70) corresponding to the Asp^{6} amino acid of the GLRaV-3 Hsp70h reduced the k_{cat} to approximately 1% of that of the WT protein. A mutation in the Hsc70 protein corresponding to Glu^{174} of GLRaV-3 Hsp70h reduced the k_{cat} to approximately 10% of that of the WT protein and a mutation in the Hsc70 protein corresponding to Asp^{197} of GLRaV-3 Hsp70h reduced the k_{cat} to approximately 1% of that of the WT protein (Wilbanks *et al.*, 1994). Single mutations in the immunoglobulin-binding protein (BiP) 70 kDa heat shock protein amino acids corresponding to the Thr^{10} and the Glu^{174} of the GLRaV-3 Hsp70h significantly inhibited the ATPase activity of this protein. There have been no reports on the effect of combining these mutations in one dysfunctional protein. It is hypothesised that the combination of these four mutations would have a cumulative effect, reducing the ATPase activity to an even greater extent and bringing it close to zero. It is further hypothesised that the mutated form of the GLRaV-3 Hsp70h protein engineered in this study is not simply a dysfunctional form of the GLRaV-3 Hsp70h MP, but that it rather can be termed a non-functional form of the protein with little or no ATPase activity. These assumptions, however, remain unproven.

It was not possible to determine whether the resistance attained in the transgenic plant lines M5 and M10 were due to RNA- or protein-mediated mechanisms. However, due to the high mRNA transcript levels noted in the Northern hybridisation for plant lines M5 and M10, it is believed that the resistance is protein mediated. RNA-mediated resistance mechanisms have very low mRNA transcript levels (Baulcombe, 1999; Moreno *et al.*, 1998; Rovere *et al.*, 2002; Sijen *et al.,* 1996; Smith *et al.*, 1994) due to the targeting of homologous RNA for degradation. Further support for protein-mediated resistance mechanism comes from the low level of homology between the GLRaV-3 *hsp70h* gene and the PVX genome. RNA-mediated resistance mechanisms require high levels of homology for transcript interaction with the viral genome and target-specific dsRNA degradation (Grant, 1999). If the resistance was due to an RNA-mediated mechanism, however, it would also be expected that there would be no difference between plants containing the *wt-hsp* and *mut-hsp* transgenes, particularly as these transgenes are essentially identical at the

nucleotide level, with only four nucleotide differences in 1650 bp. This was not observed in our experiments. Furthermore, in all cases of MPMR, the accumulation of dysfunctional MP has been required for resistance against heterologous and unrelated viruses (Beck *et al.*, 1994; Cooper *et al.*, 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.,* 1997; Tacke *et al.,* 1996). The only reported case of RNA-mediated resistance with the MP resulted in resistance against the homologous virus only (Tacke *et al.*, 1996).

Lack of nucleotide homology between the GLRaV-3 *hsp70h* gene and the genome of PVX, absence of resistance with the *wt-hsp* transgene, high mRNA transcript levels in the resistant plant lines M5 and M10 and the requirement of dysfunctional MP accumulation for resistance against unrelated viruses led us to conclude that the resistance obtained in the M5 and M10 plant lines containing the *mut-hsp* transgene is protein based. This protein-based resistance would follow the mechanism of a dominant-negative mutant in which the mutated protein is able to outcompete the WT protein and thus interferes with a normal process required for virus infection (Beachy, 1997; Beck *et al.*, 1994; Cooper *et al.*, 1995; Lapidot *et al.*, 1993). It is unknown whether the resistance obtained in these experiments is as a result of interference with the movement of PVX or due to some other process required for virus infection for which Hsp70h could be co-opted by PVX. The BYV Hsp70h has been linked to virus assembly and the movement of BYV. Cellular Hsp70s have also been linked to virus assembly, virus replication and the translocation of particular viral proteins (Cripe *et al.*, 1995; Glotzer *et al.*, 2000; Hu *et al.*, 1997; Sullivan and Pipas, 2001). Therefore, the possibility that the dysfunctional GLRaV-3 Hsp70h may interfere with any of these processes also exists with PVX. It is, however, unknown whether PVX co-opts or uses cellular Hsp70s for viral replication, assembly or virus movement. There is growing evidence that large viruses may require extra energy, provided by cellular Hsp70s or ATPase domains in viral-encoded proteins, for virus movement (Alzhanova *et al.*, 2001; Carrington *et al.*, 1998; Donald *et al.,* 1995, Soellick *et al.*, 2000) and it is possible that PVX may also require extra energy for movement. If this is proven to be true, the dysfunctional GLRaV-3 Hsp70h with reduced ATPase activity would outcompete the cellular Hsp70s that PVX would normally use for movement.

Beck *et al.* (1994) and Seppänen *et al.* (1997) showed that the movement protein-mediated resistance of viruses that use the triple gene block movement strategy could confer resistance against unrelated viruses that also use this triple gene block mechanism of movement, but could not provide resistance against viruses that did not use a similar mechanism of movement. Members of the *Closteroviridae* appear to use a similar triple gene block mechanism of movement (Alzhanova *et al.*, 2000). It is therefore conceivable that resistance obtained by the dysfunctional GLRaV-3 Hsp70h against PVX, which utilises the triple gene block mechanism of movement, will not be as broad as originally expected. To determine whether plants containing the dysfunctional GLRaV-3 Hsp70h transgene can provide

resistance to other viruses that do not utilise the triple gene block movement strategy, the M5 and M10 plant lines needs to be challenged with other viruses that do not utilise this movement strategy.

The ultimate goal of this project is to provide resistance against the viruses associated with grapevine leafroll. The results that we have presented indicate that expression of the *mut-hsp* gene can provide resistance against an unrelated virus that utilise a triple gene block movement mechanism. Speculation that members of the *Closteroviridae* also use a similar mechanism of movement, albeit with six different virus proteins (Alzhanova *et al.*, 2000; Peng *et al,* 2001), should therefore result in resistance against members of the *Closteroviridae* associated with grapevine leafroll disease.

Movement protein-mediated resistance usually results in low levels of resistance. However, Beachy (1997) postulated that increased knowledge of the structure and function of plant virus MPs would lead to the development of better mutant proteins that would provide better levels of resistance. This has been demonstrated by Tacke *et al.* (1996), who obtained 70 to 90% resistance against unrelated viruses with one of their dysfunctional MP constructs. Other dysfunctional MP constructs had lower levels of resistance (Tacke *et al.*, 1996). Results obtained in this study concur with the statement by Beachy (1997). These results show that a mutated form of the GLRaV-3 Hsp70h MP containing mutations that significantly reduce ATPase activity (Gaut and Hendershot, 1993; Wilbanks *et al.,* 1994) and abolish virus movement of the homologous virus (Peremyslov *et al.*, 1999) can be used to obtain very good levels of resistance against an unrelated virus.

The results obtained in this project will hopefully be the stimulus for studies designed to answer many of the questions that have arisen in this study. Some of our results are inconclusive, therefore, some of the conclusions we propose are derived from the theories that have been developed by other researchers that have used similar mechanisms of engineered resistance (Beck *et al.*, 1994; Cooper *et al.*, 1995; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Seppänen *et al.,* 1997; Tacke *et al.,* 1996) and fit our results. To prove the speculative conclusions of this work and establish a definitive mechanism of resistance, the following issues need to be addressed:

1. Determine whether plant lines M5 and M10 exhibits resistance against other unrelated viruses that do not use the triple gene block mechanism of movement.
2. Determine whether the resistance mechanism is RNA or protein mediated. Virus challenge of the W and M plant lines that have low transgene mRNA transcript levels (M3, M15, M16, W10, and W15) or plants that have high transcript levels (M13) can be used to determine whether resistance is observed in one or the other set of plants. Protein-mediated resistance mechanisms would only be observed in the M plant lines. Protein detection by western blot analysis or protein purification can also be used to show the presence of proteins in plants

that show a resistant phenotype but that would be absent in susceptible transgenic plants.

3. Determine whether the resistance mechanism is based on the movement of viruses or on other processes in which Hsp70 proteins can be co-opted for other functions in virus infection, such as virus assembly and replication. This could be determined by virus infection in protoplasts from plant line M5 or M10, followed by electron microscopy of the infected protoplasts. The presence of intact virions would indicate that the virus could replicate and assemble in cells expressing the mutated form of the GLRaV-3 Hsp70h. This would in turn imply that the Hsp70h interferes with the movement process of the viruses. The presence of virus RNA and no virion particle would indicate that viruses could replicate but not assemble. The absence of virus RNA and virion particles would indicate that the virus could not replicate.
4. Determine whether resistance against the GLRaVs can be obtained in grapevine.

In conclusion, this project has achieved its primary goal. A mutated copy of the *grapevine leafroll-associated virus-3* Hsp70h MP was transformed into tobacco plants. Two tobacco plants containing a mutated form of the GLRaV-3 Hsp70h MP exhibited resistance against the unrelated PVX. This result has proven the concept that a mutated form of the GLRaV-3 Hsp70h expressed in plants can provide resistance against an unrelated systemically infective plant virus. However, it remains to be seen whether this movement protein-mediated resistance strategy will provide resistance against the grapevine leafroll-associated viruses, in particular the homologous GLRaV-3 in transgenic grapevine. This will be the focus of further studies.

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