A real-time RT-PCR assay for the detection and quantification of grapevine leafroll-associated virus 3 (GLRaV-3) in *Vitis vinifera* (Vitaceae) and *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae)

By

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DECLARATION

I, Nicoleen Smit, declare that the thesis which I hereby submit for the degree of Magister Scientiae at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Signature

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Summary

Viral diseases represent a major obstacle to commercial growing of grapevines. *Grapevine leafroll-associated virus 3* (GLRaV-3) is one of the most economically important viral diseases in South African vineyards. The most important vector of GLRaV-3 in South Africa is the vine mealybug *Planococcus ficus*. Studies show that single first instar-nymphs can successfully transmit GLRaV-3 to healthy grapevines. Quantifying the amount of virus acquired in relation to feeding time of mealybugs may lead to a better understanding of GLRaV-3 mealybug transmission. In this study, a real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay was developed to quantify GLRaV-3 in grapevines and mealybugs. Real-time qRT-PCR is a highly sensitive technique. The quality of purified RNA and the accuracy of the quantification technique are essential for reliable quantitative use. To develop a real-time qRT-PCR assay two tasks were undertaken. Firstly, four different RNA isolation techniques, namely phenol-chloroform, Gentra Purescript® RNA Isolation kit, Qiagen QIAzol™ and Qiagen RNeasy® Plant Mini kit, were assessed for use in qRT-PCR. Phenol-chloroform RNA extraction method was superior to the other RNA extraction methods tested, with regard to purity of RNA, RT-PCR efficiency, reproducibility and number of GLRaV-3 positive samples detected. Secondly, DNA and cRNA external standard models were designed for quantifying GLRaV-3 in grapevines and mealybugs. External standards have defined concentrations of a target nucleic acid and are used to generate a standard curve. The DNA standard model had a wider detection and quantification range than the cRNA standard model. However, the DNA standard model, unlike the cRNA standard model is not subjected to the RT step. Therefore,

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using the DNA standard model, GLRaV-3 is quantified based on the starting cDNA concentration and not the initial RNA concentration. The cRNA standard model provides information on the initial RNA concentration. The DNA standard model is best applied as a quantification method where initial RNA concentration is not relevant. The cRNA standard model can be applied for accurate quantification of GLRaV-3 in grapevines and mealybugs. The real-time qRT-PCR assay designed in this study can be applied in other laboratories.

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CHAPTER ONE

General introduction to grapevine leafroll disease and mealybugs

1.1 Grapevine leafroll-disease

Viral diseases represent a major obstacle to commercial growing of grapevines. Grapevine leafroll disease (GLD) is one of the most economically important viral diseases in vineyards globally. Some studies show estimated yield losses of between 10 % and 70 % (Martelli, 1986; Jordan, 1993; Golino *et al.*, 2002). The disease has been reported in Europe since the early nineteenth century (Martelli, 1986). GLD occurs in all major grapevine growing regions throughout the world (Engelbrecht & Kasdorf, 1985; Martelli, 1986; Jordan, 1993; Belli *et al.*, 1994; Habili *et al.*, 1995; Krake *et al.*, 1999; Golino *et al.*, 2002). GLD has a very rapid rate of spread, allowing it to infect a new vineyard within only a few years due to natural spread of GLD and grafting of healthy vines (Krake *et al.*, 1999).

GLD causes qualitative and quantitative losses to yield and is of great concern to the wine and table grape industries (Goheen & Cook, 1959; Credi & Babini, 1997; Cabaleiro *et al.*, 1999; Golino *et al.*, 2002). The disease agent may also cause certain types of graft incompatibility and young vine failure (Golino et al., 2002). The disease is transmitted through grafting infected material and by insect vectors (Martelli, 1986; Belli *et al.*, 1994; Petersen & Charles, 1997; Krake *et al.*, 1999; Martelli *et al.*, 2002)

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1.2 Taxonomy

The disease is associated with a group of at least nine closely related closteroviruses (Alkowni *et al.*, 2004). *Grapevine leafroll-associated virus 3* (GLRaV-3), the most abundant of the leafroll-associated viruses, belongs to the family *Closteroviridae* and to the genus *Ampelovirus* (Gr. *ampelos* = grapevine) (Martelli *et al.*, 2002; Martin *et al.*, 2005; Pietersen, 2006; Akbas *et al.*, 2007). GLRaV-3 is a linear, positive-sense, single-stranded RNA (ssRNA) virus (Martelli *et al.*, 2002).

1.3 Symptoms and variability

Ampelovirus species infect monocotyledonous and dicotyledonous hosts. Leafroll-associated viruses are largely restricted to the phloem of their hosts and induce symptoms caused by secondary physiological effects, interfering with nutrient movement in the vine (Krake *et al.*, 1999; Grilli & Holt, 2000). The symptoms of GLD differ depending on environmental conditions and cultivars concerned. Leafroll symptoms first appear on the exposed leaves in the basal leaf positions of exposed shoots and may further extend increasingly outward along the shoots, or they may be confined to the leaves in mid-shoot region. Leafroll symptoms are best observed during late summer and early autumn. The most prominent symptom of leafroll is that the leaf margins roll downwards (Figs 1.1, 1.3). Depending on the cultivar, red (Fig. 1.2) or yellow (Fig. 1.3) leaf colouration can occur. Vines infected with leafroll generally have smaller canopies than unaffected vines and the infected vines have above normal acid levels, lower sugar content and are less productive, yielding grapes with lower quality (Martelli, 1986; Krake *et al.*, 1999). Rootstocks do not display leafroll symptoms and therefore the virus can easily be transmitted unknowingly through propagation and grafting. Cabernet franc, a red-berried variety, develops

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clear symptoms of the disease and, according to Martelli (1986) the symptoms may appear within one to three years after inoculation (Ling *et al.*, 2001). This cultivar is frequently used as a biological indicator for leafroll.

Fig. 1.1. Downward rolling of leaf margins

Fig. 1.2. Colouration in a red berried cultivar

Fig. 1.3. Downward rolling of leaf margins and colouration in a white berried cultivar (photo: R. Carstens).

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1.4 Photosynthate movement

Leafroll viruses are rather unevenly distributed in the plant and this results in some parts of the plant having a higher virus concentration than other parts. The uneven distribution of leafroll viruses may be a result of the route of photosynthate movement from a particular source leaf, which changes during the period of shoot growth. Photosynthate movement is upward from a newly exporting leaf to the young leaves and shoot apex. However, photosynthate movement from the same leaf is downward, with the continuing growth of the shoot and the production of new leaves (Shindy & Weaver, 1967; Quinlan & Weaver, 1970). Furthermore, application of herbicides and growth regulators may change the pattern of photosynthate movement in the grapevine (Leonard *et al.*, 1967; Shindy & Weaver, 1967). Hence the pattern of distribution of assimilates in the phloem system may be subject to changes in the relative activity, or sink capacity, of the different growth centers. Defoliation or leaf darkening may cause a reduction in the level of available assimilates within a shoot. The plant compensates for this by movement of photosynthates from neighbouring leaves (Quinlan & Weaver, 1970). Therefore, the continued changes in photosynthate movement together with the natural variability of the virus does not always make satisfactory and reproducible detection possible (La Notte *et al.*, 1997; Dovas & Katis, 2003). Furthermore, vectors feeding at different sites on the plant may be exposed to different virus loads, thus having a different probability of acquiring the virus.

1.5 Vectors and transmission characteristics

Virus transmission can occur in a persistent (circulative) or a non-persistent (stylet-borne) manner (Hohn, 2007). In the former case the virus-vector specificity is marked and only a few vector species are able to transmit the virus. Furthermore,

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lengthy acquisition and inoculation access periods (AAP & IAP) are required, viruses can be retained for long periods of time and viruses show a latent period (Ng & Falk, 2006). For non-persistently viruses, virus-vector specificity is not marked and many vector species transmit the virus. Furthermore, only brief AAP and IAP are required, virus retention is short-lived and no latent period is present (Ng & Falk, 2006). It is thought that *Ampelovirus* species have intermediate transmission characteristics (semi-persistent), between persistent or non-persistent transmission (Martelli *et al.*, 2002).

The process of virus transmission can be divided into four phases: 1) *preliminary fasting* or *feeding period*, the phase prior to acquisition feeding and the vectors may either starve or feed, 2) AAP, the period (access to feed) of vectors feeding on the virus source plant (virus acquisition), 3) *postacquisition fasting* or *feeding*, the time between the completed AAP before the start of IAP and 4) IAP, feeding of vectors on virus-free plants (virus transmission) (Roivainen, 1976).

GLRaV-3 is transmitted semi-persistently by mealybug (Pseudococcidae) and soft scale insect (Coccidae) vectors (Krake *et al.*, 1999; Petersen & Charles, 1997; Martelli *et al.*, 2002). However, a recent study suggests a circulative (persistent) transmission mechanism (Cid *et al.*, 2007). Due to its high abundance in South African vineyards, the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) is considered the most important vector of GLRaV-3 in this country. The minimum feeding times to take up and to transmit the virus is 15 minutes and 30 minutes, respectively (Krüger *et al.*, 2006).

1.6 *Planococcus ficus*

The vine mealybug *P. ficus* has a body length of approximately 4 mm, is oval shaped, segmented and is slate grey to pinkish (Fig. 1.4). *Planococcus ficus* is

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covered in a fine waxy layer, with a tassel of waxy hair-like extensions on the edges of the body (Fig. 1.4) and a thin dark stripe without wax, along the back, leaving the midline darker in colour than the rest of the body. It occurs wherever vines are grown in South Africa (Annecke & Moran, 1982; Walton *et al.*, in press). *Planococcus ficus* is present on vines in both spring and summer, feeding on the root, trunk, cordon, canes, leaves and fruit of the plants (Walton *et al.*, 2004). As the grapes ripen, mealybugs concentrate their feeding on the grape bunches. During the winter, the vines are leafless and dormant, and colonies of mealybugs occur on the stems in sheltered spots beneath the bark (Annecke & Moran, 1982).

Fig. 1.4. Adult female *P. ficus* with egg sac

It has been shown, that even a single first-instar nymph can successfully transmit GLRaV-3 to a healthy grapevine plant (Douglas & Krüger, 2008). Hence, the concentration of GLRaV-3 acquired and transmitted by a single first-instar nymph can be sufficient for successful transmission. In addition, Douglas & Krüger (2008) found in 116 instances, even with successful transmission of GLRaV-3 by mealybugs, 10 % retained the virus after an IAP of 5 days.

So far, no study has determined the amount of GLRaV-3 uptake in relation to feeding time of mealybugs, i.e. whether insects feeding on plants with different virus

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loads are equally infective. Using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), this relationship can be determined and may lead to a better understanding of GLRaV-3 transmission by mealybugs.

1.7 Research objectives and thesis outline

The main objective of this study was to develop a real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay for quantification of GLRaV-3 in grapevine and mealybugs. Specific aims were:

1) Select, fine-tune, test and compare different RNA extraction techniques using plant material and mealybugs infected with GLRaV-3 for real-time RT-PCR
2) To develop a standard curve real-time qRT-PCR assay for quantifying GLRaV-3 in grapevines and mealybugs

The thesis is written up in the form of research papers. Inevitably duplication in part of the text among chapters will occur. Chapter 2 provides a literature overview of real-time qRT-PCR. This is followed by Chapter 3 where RNA extraction methods for qRT-PCR of GLRaV-3 from grapevine and mealybugs are compared. Chapter 4 deals with the developments of a standard curve real-time qRT-PCR for GLRaV-3 in grapevines and mealybugs. Results of the previous chapters are synthesised and discussed in Chapter 5.

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CHAPTER TWO

Literature overview of real-time quantitative RT-PCR

2.1 Introduction

Reverse transcription polymerase chain reaction (RT-PCR) has been commonly used for amplifying small amounts of RNA and is widely employed for the detection of viral RNA in virus-vector related studies. For example, RT-PCR has increased the accuracy of determining the probability of disease transmission by a single mealybug vector (Douglas & Krüger, 2008), as opposed to estimates based on multiple-vector-transfer designs (Swallow, 1985). However, until development of real-time quantitative RT-PCR (qRT-PCR), RT-PCR could only provide qualitative results (Wang *et al.*, 1989).

Currently, quantification methods include northern blotting, *in situ* hybridization, ribonuclease (RNase) protection assays and qRT-PCR (Wang *et al.*, 1989; Hod, 1992; Weis *et al.*, 1992; Parker & Barnes, 1999; Bustin, 2000; Pfaffl, 2003; Bjarnadottir & Jonsson, 2005; Farrell, 2005). Northern blotting, *in situ* hybridization and RNase protection assays have rather low sensitivity (Melton *et al.*, 1984; Wang & Brown, 1999; Vu *et al.*, 2000; Fronhoffs *et al.*, 2002; Farrell, 2005), compared to real-time qRT-PCR, which is the most sensitive and flexible of the quantification techniques developed to date (Wang *et al.*, 1989; Hayward *et al.*, 1998; Freeman *et al.*, 1999; Wang & Brown, 1999; Bustin, 2000; Vu *et al.*, 2000; Gachon *et al.*, 2004; Farrell, 2005).

Real-time RT-PCR has been used for detection of *Tomato spotted wilt virus* (TSWV) in thrips (Boonham *et al.,* 2002). In potatoes, real-time RT-PCR has been used for the detection of *Potato mop top virus* (PMTV) and *Tobacco rattle virus*

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(TRV) (Mumford *et al.*, 2000) as well as *Potato leafroll virus* (PLRV), *Potato virus A* (PVA), *Potato virus X* (PVX) and *Potato virus Y* (PVY) (Agindotan *et al.*, 2007). Furthermore, real-time qRT-PCR assays have been used for detection and quantification of *Barley yellow dwarf virus* and *Cereal yellow dwarf virus* in plants (Balaji *et al.*, 2003), the MON810 event in transgenic maize (Hernández *et al.*, 2003), *Deformed wing virus* (DWV) in honeybees (Chen *et al.*, 2005), *Plum pox virus* in aphids (Olmos *et al.*, 2005), as well as *Citrus tristeza virus* in citrus plants and aphids (Ruiz-Ruiz *et al.*, 2007; Bertolini *et al.*, 2008; Saponari *et al.*, 2008).

Furthermore, real-time qRT-PCR has been applied to a broad range of studies, ranging from quantification of viruses in penaeid shrimp to bovine fecal samples (Dhar *et al.*, 2001, 2002; Fang *et al.*, 2002).

Even though real-time qRT-PCR is commonly used as a PCR method, many factors influence the outcome of the results. The type of quantification (relative or absolute), detection chemistry and instrument used all depend on the research question asked, laboratory facilities available and costs involved. Therefore, in this literature review all factors influencing real-time qRT- PCR will be discussed.

2.2 RNA extraction

Real-time qRT-PCR is a highly sensitive technique and can detect low amounts of RNA (Bustin, 2000; Gachon *et al.*, 2004). Therefore, integrity and quality of extracted and purified RNA is essential for successful and reliable diagnostic and quantitative use in real-time qRT-PCR (Ginzinger, 2002; Pfaffl, 2003; Farrell, 2005; Udvardi *et al.*, 2008). Many factors affect the quality of RNA, including phenolic compounds, polysaccharides, DNA, proteins and the enzyme RNase, all of which are well known inhibitors of RT-PCR (MacKenzie *et al.*, 1997; Wilson, 1997; Farrell, 2005). Ideally isolated RNA should be free from any PCR inhibitors. Therefore, the

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first step for studying RNA is isolating (extracting) and separating it from all other biological material in a cell. Successful isolation of intact RNA requires the following essential steps: disruption of cells or tissue, denaturation of nucleoprotein complexes, elimination or inactivation of the enzyme ribonuclease (RNase) activity and removal of unwanted DNA and proteins (Lewis, 1997; Farrell, 2005). The most important step in RNA isolation is the inactivation of RNases. Another essential step is protein removal. RNases are present in virtually any environment even within cells (Lewis, 1997; Farrell, 2005). RNases degrade RNA, thus, affecting the integrity of RNA. During cellular disruption the RNases are released from membrane-bound organelles and may mix with RNA (Lewis, 1997). Furthermore, RNases can tolerate many protein inactivation methods and are active over a broad pH range. Therefore, sample storage and extraction method can affect the quality of RNA (Lewis, 1997; Tichopád, 2004; Farrell, 2005). External sources of RNase contamination include equipment, working surfaces, some reagents, human skin and oil on fingertips (Lewis, 1997; Farrell, 2005). All these factors contribute to the difficulties in extracting intact, uncontaminated and good quality RNA. Hence, extreme precautions must be taken when handling, working with and storing RNA.

There are many extraction techniques available to overcome the difficulties in RNA isolation. These extraction techniques include the use of a combination of various chemicals such as guanidium-containing buffers, sodium dodecyl sulphate (SDS), *N*-laurylsarcosine, phenol-chloroform and isoamyl alcohol, 8-hydroxyquinoline, cesium chloride (CsCl), cesium trifluoroacetate (CsTFA), proteinase K, RNA*later*™, polyvinylpyrolidone (PVP) and mercaptoethanol (ME) to name a few (Farrell, 2005). These chemicals all assist at some level in one or more of the RNA isolation steps and are successful in the removal of PCR inhibitors.

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However, also important in RNA isolation is storage conditions. RNA should be dissolved and stored in a non-denaturing aqueous buffer, such as diethyl pyrocarbonate (DEPC) treated water at -70 ^{o}C (Farrell, 2005). DEPC is an effective inhibitor of RNase. Therefore, DEPC can be used effectively for treatment of reagents and equipment to eliminate RNase activity (Farrell, 2005).

2.3 Reverse Transcription (cDNA synthesis)

Using PCR for RNA templates requires an extra step to reverse transcribe (RT) RNA by reverse transcriptase into its single-stranded complementary DNA (cDNA) which can then be amplified (Freeman *et al.*, 1999; Bustin, 2000; Tichopád, 2004; Farrell, 2005). The RT step can be performed within the PCR assay or separately. Separating the RT and PCR step generates stable cDNA that can be stored long-term. However, performing RT and PCR in the same run reduces the risk of contamination and reduces handling time (Bustin, 2000). The reverse transcriptase enzyme *Moloney murine leukemia virus* (MMLV-RT) is commonly used in research studies. However, the *Avian myeloblastosis virus* reverse transcriptase (AMV-RT) enzyme can also be used (Freeman *et al.*, 1999; Bustin, 2000; Tichopád, 2004).

To initiate cDNA synthesis an oligonucleotide primer is required. The selected primers can either be target-specific or non-specific. Target-specific primers increase specificity and decrease non-specific binding. However, non-specific primers (random hexamers or oligo-dT primers) bind to all RNA and increase the number of mRNA molecules that can be analyzed (Freeman *et al.*, 1999; Bustin, 2000; Tichopád, 2004). Primer specificity should, therefore, be carefully considered depending on the aim of the experiment. The primer binds to RNA and during RNA-dependent DNA polymerase activity of the reverse transcriptase enzyme, the cDNA is extended toward the 5' end of the RNA. The RT step is the most variable step in the

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RT-PCR assay and is highly sensitive to low levels of PCR inhibitors that might remain in purified RNA. Therefore, the quality of purified RNA will greatly affect the synthesis of cDNA, and cDNA yield in turn affects the quantification ability of real-time qRT-PCR (Pfaffl, 2003). This once again highlights the importance of the quality of the starting material and the efficiency of the RT step for reliable qRT-PCR (Fronhoffs *et al.*, 2002; Wilkening & Bader, 2004).

2.4 Comparison of real-time RT-PCR with traditional RT-PCR

There are four phases in a PCR system, 1) the background phase, 2) the initial growth phase consisting of an exponential phase or log phase followed by 3) a linear growth phase and 4) the plateau or end-point phase (Fig. 2.1) (Kainz, 2000; Tichopád *et al.*, 2003; Tichopád, 2004).

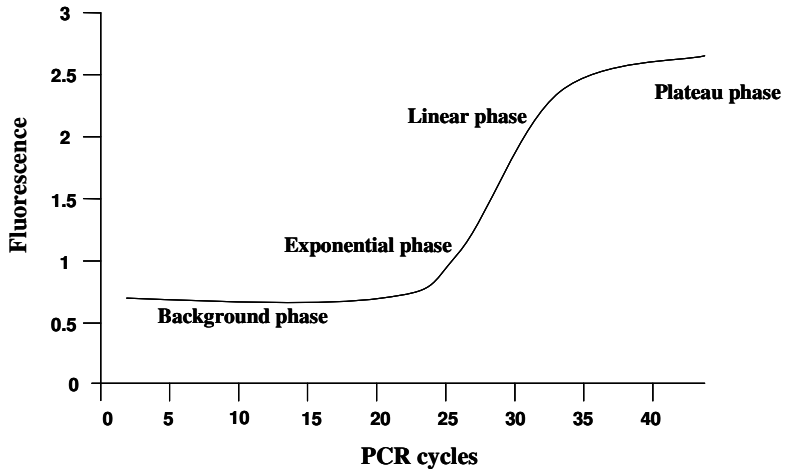

Fig. 2.1. The four phases of PCR (Modified from Pfaffl, 2003).

The background phase is the phase where amplification of template occurs, but amplicons are present below the PCR detection threshold. During the exponential phase, an increase in amplicons occurs in the form of doubling of the product that accumulates at every cycle (Wong & Medrano, 2005). The concentration of amplified product during this phase is directly related to the input template concentration

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(Schmittgen *et al.*, 2000; Pfaffl, 2003) and it is only during this phase that the starting template concentration can be determined (Ginzinger, 2002). During the linear phase there is still an increase in amplicon; however, it is much slower, as the reaction components are being consumed. The reaction stops when the plateau phase is reached, and this occurs when one or more of the PCR reagents have been depleted. Samples with different starting concentrations may reach the same plateau (Roche Applied Science, 2001). With traditional PCR, results are obtained at the plateau phase and assessment of results usually requires separation of products by gel electrophoresis. Since results are not expressed as numbers and only rely on size-based discrimination, the concentration of product in the plateau phase cannot be correlated with the initial template concentration (Tichopád, 2004). Therefore, traditional RT-PCR only provides qualitative results (Fig. 2.2).

Real-time RT-PCR applies fluorescence monitoring at the end of each cycle and enables detection and quantification of PCR products as they accumulate. Therefore, results are shown sequentially after each annealing step of each cycle of the PCR amplification process (Heid *et al.*, 1996; Bustin, 2000; Schmittgen *et al.*, 2000; Fronhoffs *et al.*, 2002; Gachon *et al.*, 2004; Wong & Medrano, 2005). Because the PCR system monitors the entire PCR reaction and not only the plateau phase it enables quantification at the exponential phase (Fig. 2.2) (Wang & Brown, 1999; Fronhoffs *et al.*, 2002; Ginzinger, 2002). As the initial template concentration can only be determined during the exponential phase, monitoring during this phase enables researchers to determine the number of cycles required first to detect a significant increase in product (Balaji *et al.*, 2003). This cycle is known as the threshold cycle (C_t) or crossing point (CP) and is only recorded during the exponential phase (Bustin, 2000). The C_t or CP is obtained when the fluorescence of the target is greater than background fluorescence and an increase in target

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fluorescence is related to initial template concentration (Ginzinger, 2002). Hence, the lower a sample's C_t or CP value the higher the starting template concentration of that sample (Ginzinger, 2002). Therefore, real-time RT-PCR is accurate and rapid, has a high sample throughput, the PCR run is considerably shorter, hands-on time is minimized and the risk of contamination is reduced (Wang *et al.*, 1989; Heid *et al.*, 1996; Fink *et al.*, 1998; Nitsche *et al.*, 1999; Bustin, 2000; Pfaffl & Hageleit, 2001; Fronhoffs *et al.*, 2002; Pfaffl, 2003; Gachon *et al.*, 2004).

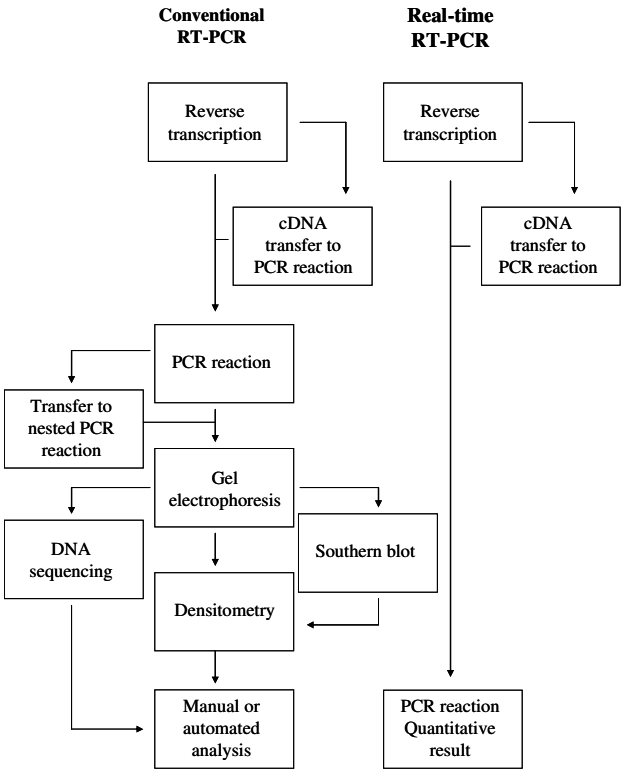

Fig. 2.2. Steps in conventional RT-PCR compared to real-time RT-PCR (Adapted from Bustin, 2000).

2.5 Quantification techniques

The two main quantification techniques that can be performed using real-time RT-PCR are absolute and relative quantification (Bustin, 2000; Ginzinger, 2002; Pfaffl, 2003; Wong & Medrano, 2005). The template, the quantity of RNA or virus present in the tissue, the accuracy level required, and whether absolute or relative

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quantification is necessary, should all be taken into consideration when determining which qRT-PCR technique to choose (Freeman *et al.*, 1999).

2.5.1 Absolute quantification

Absolute quantification allows an accurate calculation of the concentration of the target RNA in unknown samples, based on the concentration of an external standard. External standards have defined concentrations of the target nucleic acid (e.g. copy number or ng/µl), and are used to generate a standard curve for the quantification of unknown samples (Liss, 2002; Tichopád, 2004; Wilkening & Bader, 2004; Wong & Medrano, 2005). A standard curve is generated from a range of serially diluted standards that fall within the expected concentration range of the PCR products of the experimental samples (Ginzinger, 2002; Wong & Medrano, 2005). By plotting the C_t or CP values (using 95 % confidence intervals) against the logarithm of the starting copy numbers, the standard curve is created (Bustin, 2000). The external standard is amplified in the same PCR run as the sample but in a different tube or capillary (Roche Applied Science, 2003). The standard curve can be saved as a separate file. For quantitative analysis of unknown samples, the standard curve file can be imported after completion of the PCR assay. However, to enable this manner of analysis, at least one of the external standards should be included in the PCR assay. The accuracy of absolute quantification in real-time RT-PCR is completely dependent on the accuracy of the external standards; therefore the external standard curve model and the PCR efficiency of sample and standard have to be carefully determined (Freeman *et al.*, 1999; Bustin, 2000; Pfaffl & Hageleit, 2001; Fronhoffs *et al.*, 2002; Pfaffl, 2003; Gachon *et al.*, 2004; Swillens *et al.*, 2004).

The method is called absolute as it gives an absolute amount or value of target (Bustin, 2000). However, the method relies on an external standard and the term

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absolute is somewhat inaccurate (Ginzinger, 2002). Even though this method provides an actual concentration value and not a concentration measurement relative to another gene, factors such as pipetting error and RT efficiency will influence the quantification step (Farrell, 2005). Therefore, standard-curve quantification may be a more accurate description (Ginzinger, 2002).

The absolute quantification method can be applied successfully for quantifying viral load in samples (Ginzinger, 2002). The different types of external standards include recombinant RNA (recRNA), recombinant DNA (recDNA), double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), synthetic oligonucleotide and even purified RT-PCR product (Pfaffl & Hageleit, 2001; Ginzinger, 2002; Wong & Medrano, 2005). The degree of accuracy required and the purpose of the experiment will determine the type of external standard used.

2.5.2 Relative quantification

Relative quantification is more commonly used than absolute quantification (Ginzinger, 2002) as this method does not require the design of an external standard curve. Relative quantification compares the expression level (or concentration of RNA in the case of a virus) of a target gene to the expression level of a reference gene (internal control RNA) (Freeman *et al.*, 1999; Pfaffl, 2001; Ginzinger, 2002; Pfaffl *et al.*, 2002; Pfaffl, 2003; Roche Applied Science, 2001). A housekeeping gene is often used as a reference gene as it is present in all cell types (endogenous reference gene) (Kreuzer *et al.*, 1999a; Thellin *et al.*, 1999; Pfaffl, 2001). Some known housekeeping genes include tubulins, actins e.g. ß-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), albumins, cyclophilin, micro-globulins, 18S or 28S ribosomal RNA (rRNA), L32, and ubiquitin (UBQ) (Goidin *et al.*, 2001; Pfaffl *et al.*, 2004; Tichopád, 2004).

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Relative quantification can determine the changes in mRNA levels of a gene (or RNA concentration of a virus) across numerous samples. During a RT-PCR assay, the target or sample is compared directly with the reference gene and the comparison with the reference gene shows the sample to contain either more or less target than the reference (target/reference ratio) (Bustin, 2000). Ideally any reference gene should have an invariable expression level among individual samples and the PCR efficiencies of target and reference genes should be comparable (more than 90 %) (Ginzinger, 2002; Liss, 2002). This can be determined with the mathematical model of relative expression ratio described by Pfaffl (2001). The model [equation 1 (E1)] denotes the relative expression level of the target gene (R) expressed in an unknown sample versus a control in comparison to a reference gene.

$$R = \frac{(E_{tar})^{\Delta CP_{tar}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}} \qquad \text{E1}$$

E_{tar} and E_{ref} is the real-time PCR efficiency of target gene transcript (study sample) and reference gene transcript respectively, ΔCP_{target} and ΔCP_{ref} is the crossing point (CP) difference (Δ) of an unknown sample versus a control sample, of the target gene transcript (study sample) and reference gene transcript, respectively. To calculate R, the PCR efficiencies and ΔCP of all transcripts should be known. PCR efficiencies can be calculated with E2.

$$E = 10^{(-1/slope)} \qquad \text{E2}$$

To measure E a 10-fold serial dilution of the starting template is carried out and the Ct or CP value is plotted as a function of the natural logarithm concentration of the template and E is estimated from the slope of the resulting regression line. PCR reaction efficiency of 100 % is usually indicated by a slope of -3.32 (Ginzinger, 2002; Tichopád, 2004).

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2.6 Detection chemistries

A variety of fluorescent chemistries can be used to compare PCR product concentration with fluorescence intensity (Bustin, 2000; Goll *et al.*, 2006). These fluorescent chemistries can be divided into 1) sequence-independent detection assays such as DNA binding dyes (fluorescent dyes) and 2) sequence-specific probe binding assays (fluorescent probes) such as hybridization and hydrolysis probes (TaqMan probes) and molecular beacons (hairpin probes) (Wittwer *et al.*, 1997b).

Sequence-independent detection assays rely on fluorescent dyes that bind to all double-stranded DNA molecules regardless of sequence (Bustin, 2000). Fluorescent probes are sequence-specific and only detect specific PCR product (Bustin, 2000).

2.6.1 DNA-binding dyes

The simplest method for detecting and quantifying amplified product is the use of DNA-binding dyes such as Ethidium bromide (EtBr) and SYBR® Green I Dye, which are non-specific dsDNA binding fluorophores (fluorescent dyes) (Morrison *et al.*, 1998; Rasmussen *et al.*, 1998; Udvardi *et al.*, 2008). SYBR® Green I Dye gives a stronger signal than EtBr and is more commonly used (Rasmussen *et al.*, 1998). SYBR® Green I Dye fluoresces brightly when bound to dsDNA. However, during the unbound state the dye displays very little fluorescence (Fig. 2.3) (Rasmussen *et al.*, 1998; Bustin, 2000).

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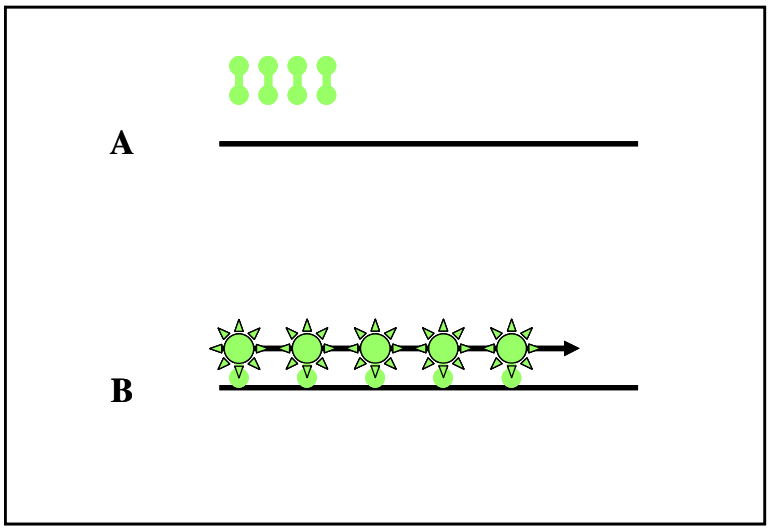

Fig. 2.3. SYBR® Green I detection format. A) No fluorescence in the presence of ssDNA and in the unbound state. B) Fluorescence in the presence of dsDNA (Modified from Roche Applied Science, 2004).

DNA-binding dyes detect target specific and non-specific dsDNA and may result in non-specific fluorescence (Rasmussen *et al.*, 1998; Bustin & Nolan, 2004). Therefore, their specificity is primer-dependent and they are less sensitive than fluorescent probes (Morrison *et al.*, 1998; Bustin, 2000). To increase specificity a melting curve analysis is included in these assays. With melting curve analysis (dissociation curve analysis), fluorescence is plotted as a function of temperature, by gradually increasing the temperature above the melting temperature (T_m) of the template and measuring the fluorescence. The T_m of the template depends greatly on its nucleotide composition, enabling identification of the signals obtained from different products during amplification (Wittwer *et al.*, 1997a; Bustin, 2000). The T_m of the template can be differentiated from amplification product (melts at lower temperatures) by a distinctive melting peak (Bustin, 2000). However, gel electrophoresis may still be necessary to ensure that the melting curve data is for a single PCR product only (Udvardi *et al.*, 2008) as shown by Wilkening & Bader

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(2004), who found two different PCR products (glutathione *S*-transferase 1 (*GSTM1*) variant 1 and variant 2 to have the same melting curve in the LightCycler even though the two products could be differentiated as two distinct bands with gel electrophoresis.

2.6.2 Hybridization probes

Hybridization probes (HybProbe) consist of two independent fluorescently labelled oligonucleotides (Caplin *et al.*, 1999; Nitsche *et al.*, 1999; Bernard & Wittwer, 2000). The probes are sequence-specific (Wong & Medrano, 2005) and a single fluorescent dye is attached to each probe (Fig. 2.4) (Wittwer *et al.*, 2001). A donor (energy transfer donor) dye is attached to the 3' end of the upstream probe and a long wavelength acceptor dye is attached to the 5' end of the downstream probe (Kreuzer *et al.*, 1999b; Nitsche *et al.*, 1999; Bustin, 2000; Wittwer *et al.*, 2001). HybProbes are not extended, because the acceptor probe is blocked (phosphorylated) at its 3' end (Nitsche *et al.*, 1999; Bustin, 2000).

After the denaturation step and during the annealing phase of PCR, both probes hybridize to the same target strand in a head-to-tail arrangement (Fig. 2.4) (Caplin, 1999; Kreuzer, 1999b; Bustin, 2000; Wittwer *et al.*, 2001; Wong & Medrano, 2005). The probes hybridize adjacent to each other and in close proximity of each other, usually between 1-5 nucleotides, with one nucleotide separation being optimal (Caplin, 1999; Wittwer *et al.*, 2001). HybProbes utilize the fluorescence (or Förster) resonance energy transfer (FRET) principle. FRET is based on energy transfer from one fluorescent molecule (fluorescein) to another neighbouring fluorescent molecule (Fig. 2.4). The efficiency of FRET is highly dependent on the spacing of the two probes when hybridized to the target sequence (Bustin, 2000). The donor probe is excited, resulting in energy transfer (FRET) to the acceptor probe and

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the acceptor probe emits fluorescent light at a different wavelength that is measured during every cycle by a real-time PCR instrument (Fig. 2.4) (Kreuzer *et al.*, 1999b; Bustin, 2000; Wittwer *et al.*, 2001). The amount of fluorescent light emitted by the acceptor probe is directly related to the starting concentration of the template. Fluorescence is plotted against cycle number as fluorescence is obtained once during each cycle (Caplin, 1999). The probes are only hybridised and not hydrolysed, therefore fluorescence can be reversed and melting curves can be generated (Bustin, 2000).

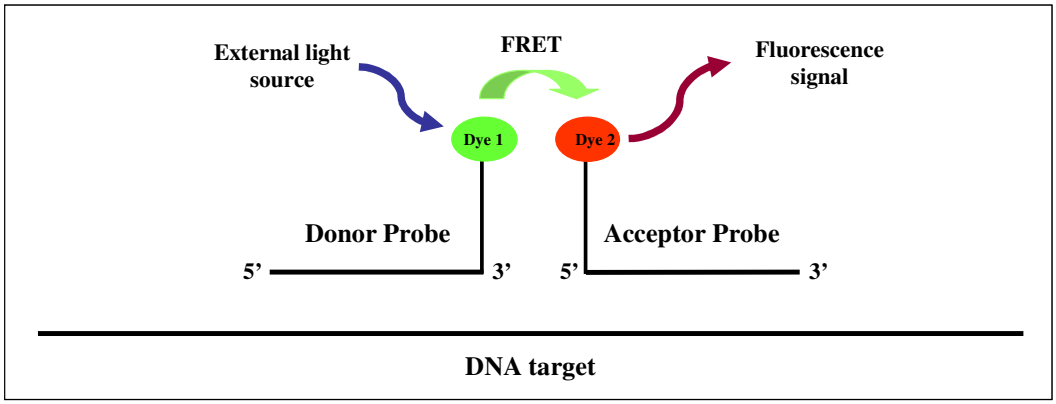

Fig. 2.4. Principles of HybProbes (Adapted from Caplin *et al.*, 1999). The donor probe is excited by an external light source resulting in FRET between the donor and acceptor probe and emission of a fluorescence signal by the acceptor probe.

2.6.3 Hydrolysis probes

Hydrolysis probes are also known as the 5' nuclease assay, exonuclease probe format or TaqMan assay. The probe is dual-labelled with a fluorescent reporter and quencher dye at the 5' and 3' ends, respectively (Fig. 2.5) (Gibson *et al.*, 1996; Kreuzer *et al.*, 1999b; Nitsche *et al.*, 1999; Bustin, 2000). Hydrolysis probes are sequence-specific and utilize the 5' exonuclease activity of the enzyme Taq DNA polymerase enabling measurements of the amount of target template in a sample.

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Before hydrolysis of the probe the two dyes remain in close proximity of each other and are separated only by the probe length. If the probe remains intact, the emission of the 5' reporter dye is quenched by the 3' quencher dye (Gibson *et al.*, 1996; Heid *et al.*, 1996; Bustin, 2000; Dötsch *et al.*, 2001; Osman *et al.*, 2007).

During the annealing and extension phase of PCR, the probe hybridizes to an internal region of the target template, between the forward and reverse primer pair. The polymerase enzyme extends the primer and the template to which the probe is hybridized is replicated. Upon reaching the probe, the 5' exonuclease activity of the polymerase enzyme hydrolysis (cleaves) the probe, releasing the reporter dye in a fork-like structure (Fig. 2.5) (Holland *et al.*, 1991; Gibson *et al.*, 1996; Bustin, 2000). The separation of the reporter dye from the quencher dye results in an increase in fluorescence intensity of the reporter dye (Fig. 2.5) (Nitsche *et al.*, 1999; Bustin, 2000; Uhl *et al.*, 2002). The increase in fluorescence intensity of the reporter dye is related to the amount of amplified product (Osman *et al.*, 2007). This procedure is repeated during each cycle and does not affect PCR product accumulation. Fluorescence is irreversible, the probes are digested and melting curves cannot be generated (Bustin & Nolan, 2004; Roche Applied Science, 2004).

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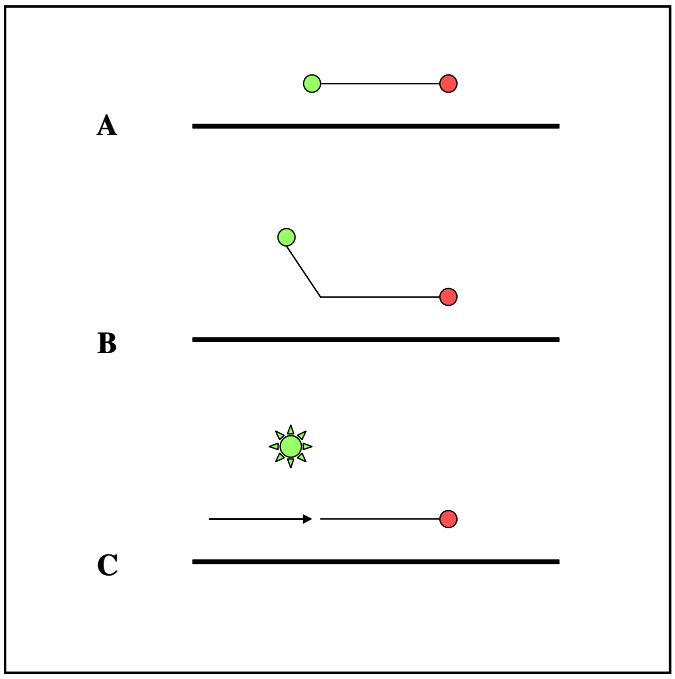

Fig. 2.5. Principle of TaqMan probes (From Cockerill, 2003). During the unbound state the fluorescence of the reporter is quenched. The 5' exonuclease activity of the polymerase cleaves the probe and the reporter dye is released, resulting in fluorescence emission.

2.6.4 Molecular beacons

Molecular beacons (hairpin probes) are sequence specific hybridization probes (Wittwer *et al.*, 2001). These probes are labelled at opposite ends with a fluorescent reporter dye and a non-fluorescent quencher dye (Fig. 2.6). (Giesendorf *et al.*, 1998; Bustin, 2000; Wong & Medrano, 2005). The two dyes on the molecular beacon remain in close proximity to each other by forming a stem-loop hairpin structure (Fig. 2.6). The stem-loop hairpin structure is only formed in the absence of the target, allowing the fluorescence of the reporter dye to be quenched (Giesendorf *et al.*, 1998; Bustin, 2000; Tsourkas & Bao, 2003). The stem is formed by annealing of two complementary arm sequences on each end of the probe sequence. The two arm sequences are not complementary to the target sequence, whereas the loop section is

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complementary to the target sequence (Giesendorf *et al.*, 1998; Bustin, 2000). When the target sequence or molecule is present the molecular beacon opens by forming a stable probe-target hybrid (Fig. 2.6), therefore increasing the distance between the reporter and quencher and restoring reporter fluorescence emission (Giesendorf *et al.*, 1998; Bustin, 2000; Tsourkas & Bao, 2003; Wong & Medrano, 2005). Therefore, molecular beacons only fluoresce when hybridized to the target (Fig. 2.6) (Bustin, 2000; Uhl *et al.*, 2002).

A molecular beacon should be carefully designed, i.e. the reporter dye should always be in close proximity to the quencher dye. Molecular beacons can fold into alternate structures, which could affect the positioning of the reporter and quencher dye allowing background fluorescence as the reporter dye cannot be quenched (Bustin, 2000). However, the fluorescence of an annealed molecular beacon may only be partially restored if the stem is too strong (Bustin, 2000).

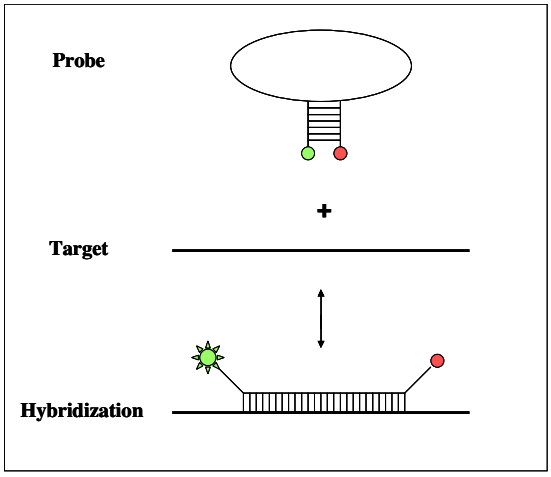

Fig. 2.6. Principle of molecular beacons (From Cockerill, 2003). The close proximity of the reporter and the quencher dye in the stem-loop hairpin structure causes the reporter fluorescence to be quenched. In the presence of the target the molecular beacon opens and forms a stable probe-target hybrid and reporter fluorescence is restored.

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2.7 Real-time PCR instruments

The main real-time PCR instruments currently available are listed in Table 2.1.

Table 2.1. Examples of commercial real-time PCR instruments (Adapted from Cockerill, 2003).

Instrument	Manufacturer	Automated Extraction	Thermal Cycling Format	Detection Format	Analytic Turn Around Time (TAT)	Data Observation	Reaction Capacity (per run)
GeneAmp 5700 and Prism 7700	Applied Biosystems	Yes (ABI PRISM 6700)	Conventional heating block thermal cycler	TaqMan or molecular beacons	Approximately 2 h	End point	96-384
iCycler	BioRad	No	Conventional heating block thermal cycler	TaqMan, FRET, or molecular beacons	Approximately 2 h	Each PCR cycle	48, 60, 96 and 384
LightCycler 1.5, 2.0 and 480 Instrument	Roche Applied Science	Yes (MagNA Pure LC)	Ambient air cooling	TaqMan, FRET, or molecular beacons	Approximately 20 min to 1 h	Each PCR cycle	32 and 480
SmartCycler	Cepheid	Planned for release (Gene-Expert)	Ceramic heating plate	TaqMan, FRET, or molecular beacons	Approximately 40 min to 1 h	Each PCR cycle	16, 32, 48, 68, 80 and 96
MX4000	Stratagene	No	Conventional heating block thermal cycler	TaqMan, FRET, or molecular beacons	Approximately 2 h	Each PCR cycle	96
Rotor Gene	Corbett Life Science	No	Ambient air cooling	TaqMan, FRET, or molecular beacons	Approximately 50 min	Each PCR cycle	32

The latest introduction from Applied Biosystems is a range of StepOnePlus™ real-time PCR systems and the StepOne™ real-time PCR system.

Eppendorf North America has also introduced the Eppendorf Mastercycler ep *realplex* ^{2} S and Eppendorf Mastercycler ep *realplex* ^{4} S (Reaction capacity = 96). Idaho Technology Inc. provides the following real-time PCR intruments R.A.P.I.D.® LT Instrument (Reaction capacity = 32), R.A.P.I.D.® System, RapidCycler® 2 Instrument, RAZOR® EX Instrument.

Because real-time PCR systems are run as closed-tube systems, no post-PCR handling is required. Therefore, the handling time is reduced which minimizes the risk of contamination. Furthermore, these closed-tube systems enable short turn-around times for data acquisition and analysis (Bustin, 2000).

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Real-time PCR instruments also provide rapid thermocycling because of the high surface-volume ratio of the PCR mix and the extremely efficient heat exchange system (temperature control system). The high surface-volume ratio of the PCR mix is due to narrow and elongated reaction cuvettes (e.g. glass capillaries). Furthermore, rapid changes in temperature around the cuvettes are generated by efficient air exchange and thermal conductivity through solid-phase material (Cockerill, 2003).

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CHAPTER THREE

Comparison of RNA extraction methods for real-time quantitative RT-PCR of *Grapevine leafroll-associated virus 3* (GLRaV-3) from grapevine and mealybugs

Abstract

The integrity of purified RNA is critical for reliable diagnostic use in real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR). Sampling, storage and extraction method affect the integrity of purified RNA. *Grapevine leafroll-associated virus 3* (GLRaV-3) has been associated with one of the most economically important viral diseases of grapevines. An important vector is the vine mealybug *Planococcus ficus*. For the development of a real-time qRT-PCR for GLRaV-3, the efficiency of four extraction methods (phenol-chloroform, Gentra Purescript® RNA Isolation kit, Qiagen QIAzol™ and Qiagen RNeasy® Plant Mini kit) was evaluated using plant material and mealybugs. The PCR results showed the phenol-chloroform method to be the most efficient, reliable and reproducible RNA isolation method. This method is cost effective and can easily be implemented in any laboratory. The QIAzol method could not be used as an RNA isolation method in this study, as it yielded a low number of GLRaV-3 positive samples.

3.1 Introduction

The quality and purity of RNA is critical for successful and reliable use in real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-

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PCR) (Bustin, 2000; Pfaffl, 2003; Gachon *et al.*, 2004). Real-time RT-PCR is highly sensitive, enabling detection of low amounts of RNA. However, sampling, storage and extraction method affect the integrity and purity of extracted RNA (Pfaffl, 2003; Tichopád, 2004). The majority of RNA extractions are contaminated with low levels of DNA and protein that interfere with the efficiency of RT-PCR (Pfaffl, 2004). Further, RNases readily degrade RNA during sampling, purification and storage, inhibiting RT-PCR and often leading to unreliable results (Freeman *et al.*, 1999; Farrell, 2005). Because of the sensitivity of real-time qRT-PCR, choosing a RNA extraction method that produces high-quality intact RNA is essential.

The most important steps in total RNA isolation include: cell lysis, RNase activity inhibition, unwanted protein removal and recovery of intact RNA (Lewis, 1997; Tichopád, 2004; Farrell, 2005). Often, the most successful RNA extraction techniques involve the use of chemicals, i.e. phenol-chloroform, mercaptoethanol (ME), polyvinylpyrrolidone (PVP) and guanidine thiocyanate (GTC) to remove compounds affecting PCR efficiency. Phenol and chloroform denature and precipitate proteins and simultaneously inactivate RNase. Chloroform stabilizes the phenol, improves protein denaturation and assists with lipid removal (Farrell, 2005). Further, acidified phenol and chloroform enable removal of DNA and proteins by separating the RNA into an upper aqueous phase and DNA and proteins into a lower organic phase. ME acts as an antioxidant and enhances protein denaturation and disruption of RNase, PVP binds phenolic compounds, and GTC denatures proteins and the enzyme RNase (Lewis, 1997; MacKenzie *et al.*, 1997; Salzman *et al.*, 1999; Nassuth *et al.*, 2000; Farrell, 2005). RNA extraction techniques which make use of these chemicals are effective in removal of PCR inhibitors, but are also considered the most challenging and are often lengthy and difficult (Lewis, 1997; Nakaune & Nakano, 2006).

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Grapevine leafroll-associated virus 3 (GLRaV-3) occurs in all grapevine-growing regions throughout the world and causes one of the most economically important viral diseases of grapevine (Martelli, 1986). The virus is transmitted through grafting and by mealybug (Hemiptera: Pseudococcidae) and scale insect (Hemiptera: Coccidae) vectors (Tanne *et al.*, 1989; Engelbrecht & Kasdorf, 1990; Belli *et al.*, 1994). One of these insect vectors is the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) (Engelbrecht & Kasdorf, 1990; Douglas & Krüger, 2008).

GLRaV-3 (family *Closteroviridae,* genus *Ampelovirus*) is a linear, positive-sense, single-stranded RNA (ssRNA) virus and belongs to a group of at least nine closely related viruses (Martelli *et al.*, 2002; Alkowni *et al.*, 2004). GLRaV-3 is rather unevenly distributed in the plant, resulting in certain parts of the plant manifesting higher virus concentrations than others; thus satisfactory and reproducible detection is not always possible (La Notte *et al.*, 1997). In addition, grapevine is a woody plant and is rich in phenolic compounds (Minafra & Hadidi, 1994) and polysaccharides, which are recognized PCR inhibitors (MacKenzie *et al.*, 1997; Wilson, 1997).

Several extraction methods have been used for detection of GLRaV-3 (e.g. Minafra & Hadidi, 1994; La Notte *et al.*, 1997; MacKenzie *et al.*, 1997; Acheche *et al.*, 1999; Nakaune & Nakano, 2006). In addition, a number of commercial kits are available that are rapid, simple and efficient in extracting total RNA and effectively remove PCR inhibitors (Lewis, 1997; Read, 2001; Farrell, 2005). The RNA extraction method selected for real-time qRT-PCR affects the integrity, quantity and quality of RNA. In the current study different RNA extraction methods, using GLRaV-3 positive plant material and mealybugs, were compared for real-time qRT-PCR.

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3.2 Material and Methods

3.2.1 Plants

Grapevine cv. Cabernet franc (CF) plants and a single plant propagated from stem cuttings of the rootstock hybrid LN33 (1/5/2, ARC Infruitec/Nietvoorbij), served as virus source for GLRaV-3. The virus source plants (all with the single known virus isolate) were tested before experiments with nested RT-PCR using the external and internal primer sets and a protocol adapted from Ling *et al.* (2001) (Douglas & Krüger, 2008) to confirm their virus status.

3.2.2 Insects

A non-viruliferous culture of *P. ficus,* with specimens obtained from grapevine in the Western Cape (South Africa) and established on butternut (*Cucurbita moschata* Duchesne (Cucurbitaceae)), a virus non-host, was used. The identification of *P. ficus* was confirmed using a multiplex PCR developed to distinguish three mealybug species associated with grapevine (Saccaggi *et al.*, 2008). Sub-samples of *P. ficus* (n = 20) were tested for GLRaV-3 with nested RT-PCR (Ling *et al.*, 2001; Douglas & Krüger, 2008) to confirm their virus-free status.

3.2.3 Sampling and RNA extraction methods

Different methods for RNA extraction from samples of both GLRaV-3 positive grapevine leaves and mealybugs were tested. The extraction methods tested included the phenol-chloroform method (Total RNA extraction from *Arabidopsis* and tobacco Allison C. Mallory, Bartel Lab Whitehead Institute (http://web.wi.mit.edu/bartel/pub/protocols/Total%20RNA%20extraction.pdf)), Gentra Purescript® RNA Isolation Kit and Qiagen QIAzol™ extraction method.

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Planococcus ficus nymphs were transferred from butternut to virus source (CF) plants for an acquisition access period (AAP) of 1 to 4 days. After the AAPs, the mealybugs were collected together with the leaf area (in the form of a leaf punch) where the mealybugs had been feeding was sampled as control (Fig. 3.1a,b). A leaf punch resulted in a sample of approximately 2 mm in diameter (Fig. 3.1b). The samples were taken in the petiole region of the leaves, as this is the area where GLRaV-3 concentration is thought to be the high (Ling *et al.*, 2001). Samples were randomly allocated to each extraction method. Twenty-five mealybugs and 25 leaf punches taken from these associated feeding sites were collected for each extraction method/kit. Samples were stored at -70 ^{o}C.

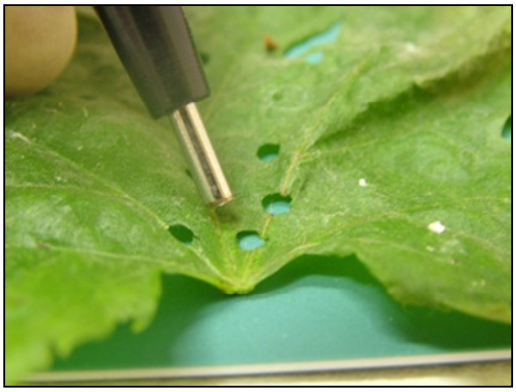
a)  b) 

Fig. 3.1. Sampling techniques, a) sampling of a single *P. ficus* nymph and b) leaf punch sample (2 mm diameter) from which *P. ficus* nymph was collected.

3.2.4 Standardization

The initial steps in sample preparation for each extraction method were standardized. For each extraction method leaf punches and their associated mealybug nymphs were taken from -70 ^{o}C and immediately placed in liquid nitrogen, to avoid thawing of the samples. The samples were ground with pestles, whilst kept in liquid nitrogen. The samples were placed on ice and ground further. Thereafter, the first

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reagent for each protocol assessed was added to the samples and the samples were homogenized.

Samples were homogenized for 1 min using a Soniprep 150 Ultrasonic disintegrator (Optolabor (Pty) Ltd). Between each 20 seconds of the 1 min, there was a 10 second interval. The samples were further homogenized by aspirating them 20 times through a P20 pipette.

After the completion of each extraction protocol, each sample was re-suspended in 14 µl diethyl pyrocarbonate (DEPC) treated water, the samples were flash frozen with liquid nitrogen and stored at -70 ^{o}C for later analysis using RT-PCR.

The total RNA concentration of every extracted sample was measured on a Biowave S2100 UV/Vis Diode Array Spectrophotometer (Biochrom Ltd) or a Nanodrop ND-1000 Spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd). If the total RNA concentration of a sample was more than 50 ng/µl the sample was diluted to 25 ng/µl and 2 µl (50 ng) was used for RT-PCR. If the total RNA concentration of a sample was less than 50 ng/µl, a calculation was done to determine the volume of sample to be used for RT-PCR, to ensure that the final amount of the sample added to the PCR mixture was 50 ng.

Extraction methods were tested using conventional nested RT-PCR (Ling *et al.*, 2001; Douglas & Krüger, 2008). PCRs for each extracted sample were repeated three times using a 2720 Thermal Cycler (Applied Biosystems). PCR products were visualized under ultraviolet (UV) light on a 1.5 % agarose gel stained with ethidium bromide (EtBr) or a 2 % agarose gel stained with GoldView™ (SBS Genetech) nucleic acid stain.

For the phenol-chloroform method the RNA extraction buffer (100 µl per sample) was added to each sample for the homogenization step. The total RNA extraction protocol from *Arabidopsis* and tobacco described by Allison C. Mallory,

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Bartel Lab Whitehead Institute was followed (http://web.wi.mit.edu/bartel/pub/protocols/Total%20RNA%20extraction.pdf) (Appendix B).

For the Gentra method the Cell Lysis solution (300 µl per sample) was added to the samples for cell lysis and the homogenization step. The manufacturer's specifications were followed (Appendix B).

For the QIAzol method the QIAzol reagent (100 µl per sample) was added to each sample for the homogenization step. RNA extractions were done according to the manufacturer's specifications with the following minor adaptations: after adding the chloroform the samples were vortexed for 1 min, if a centrifugation time was between 5-10 min, the longer centrifugation time was chosen and the RNA was not further purified (Appendix B).

3.2.5 Modification of QIAzol RNA isolation

Initially, the results for the QIAzol extractions were very poor compared to other extraction methods. However, in view of the advantages of cost and time of the extraction method, it was decided to use a clean-up method to test whether the results of the QIAzol extractions could be improved. The method used included adding different percentages of PVP-40 and β-mercaptoethanol (β-ME) to the QIAzol reagent before proceeding with extraction.

Leaf punches from grapevine cv. Cabernet franc (CF) were collected and the QIAzol extraction procedure described above was followed.

Based on Mackenzie *et al.* (1997), the following concentrations of PVP-40 and β-ME: 1 %, 2.5 % and 5 % (wt/vol) PVP-40, in combination with 1 % or 2 % (vol/vol) β-ME, and each of the PVP-40 and β-ME concentrations on their own were added to QIAzol to test whether the extraction efficiency of QIAzol in combination

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with these two chemicals could be improved. A total of 45 leaf punches were taken from CF, i.e. three samples per combination/concentration. Twelve samples served as controls, six of which were extracted using the original QIAzol extraction method and six samples using the Gentra extraction kit. The extractions were tested using nested RT-PCR.

Based on the PCR results using the different combinations of PVP-40 and β-ME (Table 3.1), the QIAzol and Gentra controls and only combinations of β-ME and PVP-40 percentages added to QIAzol, except the 5 % PVP-40 combination, were further tested using 10 leaf punches per method/combination (Fig. 3.2).

Table 3.1. Nested RT-PCR results for different concentrations of ß-Mercaptoethanol and PVP-40 added to the QIAzol reagent[a].

	ß-Mercaptoethanol		PVP-40			ß-Mercaptoethanol and PVP-40					
Percentage of chemical added to QIAzol reagent	1%	2%	1%	2.50%	5%	1%;1%	1%;2.5%	1%;5%	2%;1%	2%;2.5%	2%;5%
Number of samples tested	3	3	3	3	3	3	3	3	3	3	3
Positive samples	0	1	0	0	0	2	1	1	1	2	1
Percentage Positive	0	33.33	0	0	0	66.66	33.33	33.33	33.33	66.66	33.33

[a]None of the QIAzol control RNA extractions (n = 6) tested positive for GLRaV-3. However, 50 % of the Gentra control RNA extractions (n = 6) tested positive for GLRaV-3.

The combination of β-ME and PVP-40 with the QIAzol reagent resulted in a higher number of positive samples than the QIAzol reagent on its own (Fig. 3.2). Therefore, the QIAzol RNA isolation was repeated and replicated (25 leaf punches, 25 mealybugs), following the sampling protocol described above and the modified QIAzol extraction method using an additional 1 % β-ME and 2.5 % PVP-40, with the QIAzol reagent.

As a control, the Gentra RNA isolation was also repeated. Even though the Gentra extraction kit was withdrawn from the market, after tests were completed, the

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remainder of our kit (n = 26; 13 leaf punches, 13 mealybugs) was sufficient to use as a control.

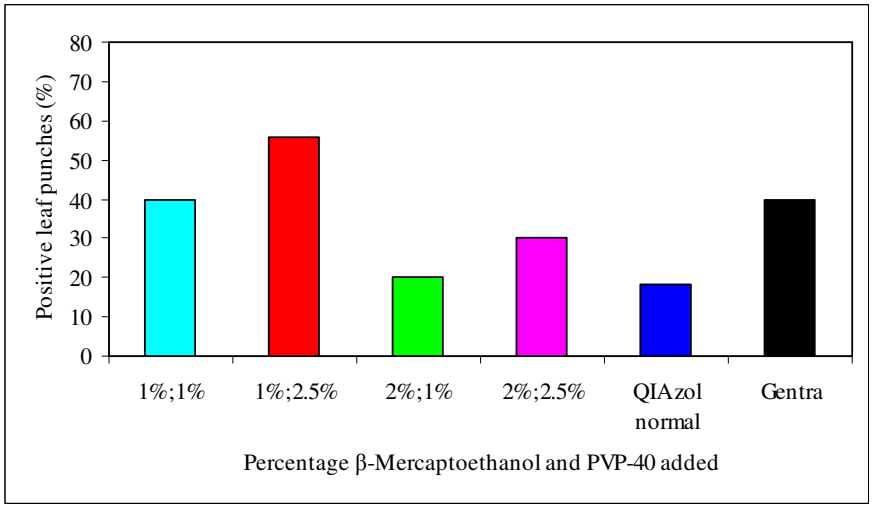

Fig. 3.2. Nested RT-PCR results for different concentrations of β-ME and PVP-40 added to the QIAzol reagent based on 10 leaf punches per method. QIAzol normal and Gentra extractions served as controls.

3.2.6 Sampling and RNA extraction for comparison of real-time RT-PCR efficiency

To compare the PCR efficiencies of the phenol-chloroform method with the Gentra and QIAzol-adjusted extraction methods and with the QIAzol-adjusted and Qiagen RNeasy Plant Mini kit using real-time RT-PCR, additional samples were collected in such a manner as to ensure the highest standardization possible.

Leaf punch samples were taken along the three main veins from leaves of a single CF plant. Two randomly selected leaf punches per vein were placed into one of three collection tubes, equaling a total of six leaf punches per collection tube (Fig. 3.3 a,b). This resulted in one sample/tube per extraction method, per leaf. A total of 12 CF leaves were used to obtain six samples per method. The sampling technique was repeated for mealybugs. However, mealybugs were randomly selected from the leaf as they were not always situated in the desired sampling area.

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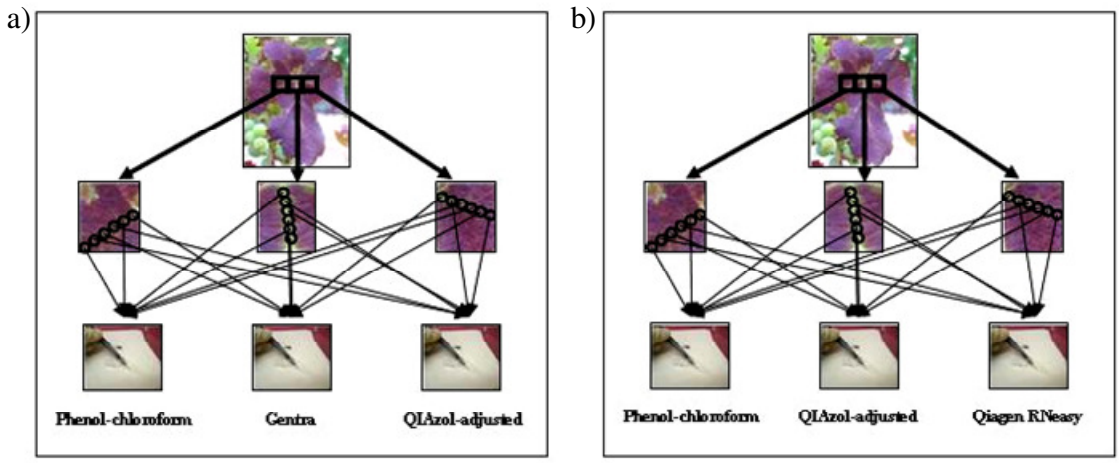

Fig. 3.3. Sampling techniques for comparing the PCR extraction efficiency of the phenol-chloroform method with a) the Gentra and QIAzol-adjusted extraction methods and b) the QIAzol-adjusted and Qiagen RNeasy Plant Mini kit.

For phenol-chloroform, QIAzol-adjusted and Qiagen methods a total of six leaf and six mealybug samples were tested. Unfortunately, only three leaf and three mealybug Gentra samples could be tested, as there was not enough of the kit left to complete a total of six replicates.

The Qiagen RNeasy® Plant Mini Kit, which is commonly used for viral RNA extraction from plant material for real-time PCR, was included as a standard commercial extraction kit, because the Gentra kit had been removed from the market. The Qiagen kit has been successfully used for GLRaV-3 extractions (Nassuth *et al.*, 2000; Osman *et al.*, 2007; Osman *et al.*, 2008). In the current study, the Qiagen kit was compared with the phenol-chloroform and the QIAzol-adjusted method.

For the Qiagen kit the same standardization process described previously was followed. Buffer RLT (450 µl) was added to each sample for the homogenization step. RNA extractions were done according to the manufacturer's specifications. The RNA was not further purified (Appendix B).

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3.2.7 Comparisons of real-time RT-PCR efficiency

Real-time RT-PCR tests were done to ensure the selection of the most efficient extraction method. Real-time RT-PCR was performed using LightCycler® TaqMan® Master kit, the LightCycler® instrument (Roche Applied Science) and the protocol adapted from Osman & Rowhani (2006) and Osman *et al.* (2007). The 56 F, 285 R and 181 P primers and probe designed by Osman & Rowhani (2006) were used. The 181 P probe was labeled at the 5'-end with the fluorescent dye FAM as reporter and at the 3'-end with BHQ-1 fluorescent dye as quencher. The primer/probe mixture (GLRaV-3 primer/probe mixture) was as follows: 20 µl each of the 100 pmol/µl forward (56 F) and reverse (285 R) primers and 4 µl of the 100 pmol/µl TaqMan® probe (181 P) were added to 196 µl SABAX water to bring the final volume to 240 µl (Osman *et al.*, 2007). The reaction mixture contained 12.75 µl water (PCR grade), 0.6 µl of the GLRaV-3 primer/probe mixture, 18 u HPRI RNase inhibitor, 40 u M-MLV reverse transcriptase, 4 µl TaqMan® Master Mix and 0.5 µl – 4 µl (50 ng) RNA extraction. The reverse transcription and amplification reaction conditions were 45 ^{o}C for 35 min, 95 ^{o}C for 10 min, followed by 60 cycles of 95 ^{o}C for 15s, 60 ^{o}C for 1 min and 72 ^{o}C for 1s. The PCR products were visualized under UV light on a 2 % agarose gel stained with GoldView™ (SBS Genetech) nucleic acid stain, to confirm results obtained from LightCycler® software 4.0.

The first extraction efficiency comparison tests were done using the single leaf punch and single mealybug samples that tested positive in all three replicates of the conventional RT-PCR. The samples were randomly selected from all the phenol-chloroform, Gentra, QIAzol-adjusted and Gentra control extraction methods. A total of 28 samples were selected, seven per extraction method, comprising of both plant

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and mealybug samples[1]. The extraction efficiency of the phenol-chloroform method was compared to that of the Gentra and the QIAzol-adjusted to that of the Gentra control. The comparisons were done in this manner because the samples collected for the phenol-chloroform and Gentra extractions, and the QIAzol-adjusted and Gentra control extractions were from the same leaves, respectively.

The second extraction efficiency comparison tests were done using the samples collected specifically for extraction efficiency comparisons. First the phenol-chloroform, Gentra and QIAzol-adjusted extraction efficiencies were compared, followed by the phenol-chloroform, QIAzol-adjusted and Qiagen extraction efficiencies.

3.3 Results and Discussion

Testing samples prepared from woody plants with PCR is difficult due to the presence of PCR inhibitors (Osman & Rowhani, 2006) such as phenolic compounds, polysaccharides and carbohydrates. This is reflected in the poor results obtained with the QIAzol method. Even though the total RNA concentrations of the QIAzol extractions in this study were the highest of all three extraction methods (some readings were over 1000 ng/µl), the QIAzol extractions yielded fewer positive samples (40 % leaf punches; 24 % mealybugs) than the phenol-chloroform (64 % leaf punches; 64 % mealybugs) or Gentra (84 % leaf punches; 68 % mealybugs) extractions for both leaf samples and mealybugs (Fig. 3.4a). In the present study, reproducibility of the extraction methods was tested with replicate PCRs of each extraction sample. The phenol-chloroform (52 % leaf punches; 24 % mealybugs) and Gentra (52 % leaf punches; 28 % mealybugs) extractions proved to be more

[1]Only seven samples per extraction method could be used due to the small sample volumes (14 µl), the standardization of the amount of sample (50 ng) used for each RT-PCR and the number of QIAzol-adjusted samples that tested positive with all three nested RT-PCRs

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reproducible than the QIAzol extractions which fared poorly (4 % leaf punches; 0 % mealybugs) (Table 3.2). Interestingly, Chomczynski (1993) showed a solution of phenol and guanidine thiocyanate, the basis of the QIAzol reagent (Qiagen QIAzol™ Handbook, January 2003), to be successful for total RNA extraction from rat thymus, human mammary epithelial cells and rat mammary tissue being effective in producing a high yield of RNA, free from PCR contaminants (Chomczynski, 1993; Liu *et al.*, 1998). However, the type of sample (plant, insect or mammal) used for RNA extraction may determine RNA extraction ability and efficiency of QIAzol.

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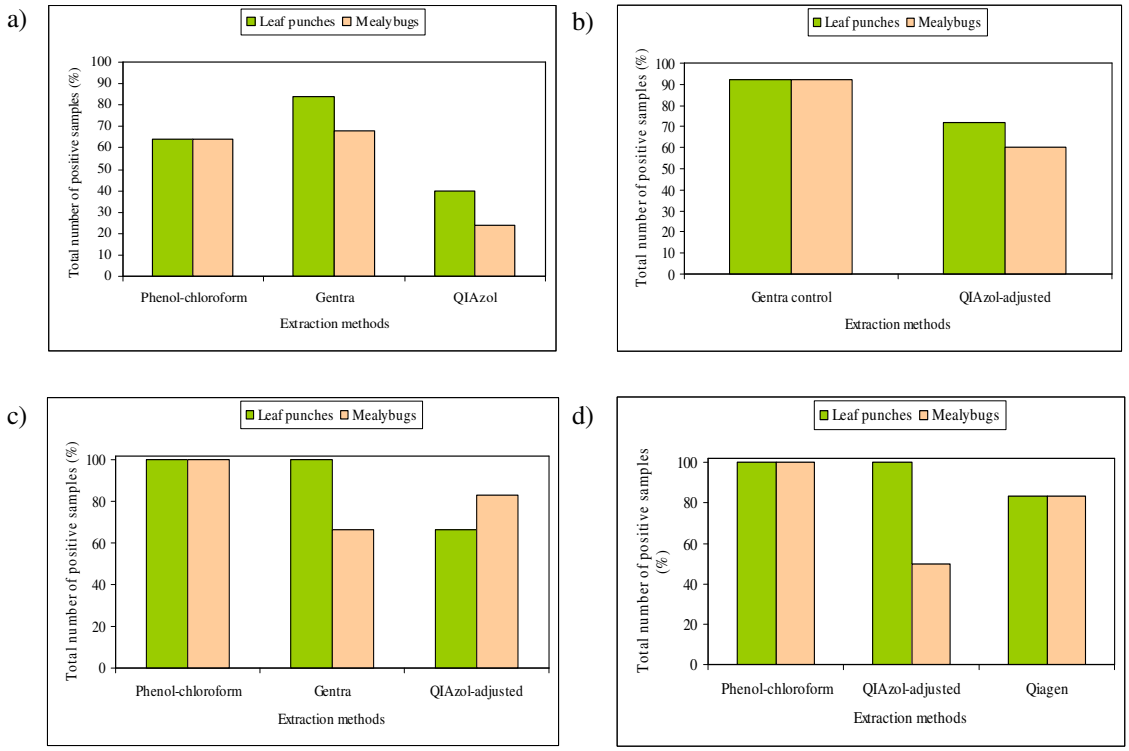

Fig. 3.4. Percentage of positive leaf punch and mealybug samples from different RNA extraction methods; a) nested RT-PCR results for three different methods (leaf punch: n = 25; mealybugs: n = 25), b) nested RT-PCR results for QIAzol-adjusted (leaf punch: n = 25; mealybugs: n = 25) and Gentra control methods (leaf punch: n = 13; mealybugs: n = 13), c) real-time RT-PCR extraction efficiency results, for phenol-chloroform and QIAzol-adjusted (leaf punch: n = 6; mealybugs: n = 6), Gentra (leaf punch: n = 3; mealybugs: n = 3) methods and d) real-time RT-PCR extraction efficiency results (leaf punch: n = 6; mealybugs: n = 6).

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Table 3.2. Nested RT-PCR reproducibility of positive leaf punches and mealybugs for different extraction methods, using the same extract and repeating the PCR three times on different days[a].

	Leaf punches														
	Phenol-chloroform (n = 25)			Gentra (n = 25)			QIAzol (n = 25)			Gentra control (n = 13)			QIAzol-adjusted (n = 25)		
Times sample tested positive	1x	2x	3x	1x	2x	3x	1x	2x	3x	1x	2x	3x	1x	2x	3x
Positive samples	3	0	13	3	5	13	7	2	1	1	3	8	8	4	6
Percentage positive	12	0	52	12	20	52	28	8	4	7.69	23.1	62	32	16	24
	Mealybugs														
	Phenol-chloroform (n = 25)			Gentra (n = 25)			QIAzol (n = 25)			Gentra control (n = 13)			QIAzol-adjusted (n = 25)		
Times sample tested positive	1x	2x	3x	1x	2x	3x	1x	2x	3x	1x	2x	3x	1x	2x	3x
Positive samples	7	2	6	4	3	7	6	0	0	1	1	10	9	5	1
Percentage positive s	28	8	24	16	12	28	24	0	0	7.69	7.69	7.7	36	20	4

[a]1x – The same RNA extract only tested positive with one of the three nested RT-PCRs. 2x – The same RNA extract tested positive with two of the three nested RT-PCRs. 3x – The same RNA extract tested positive with all three nested RT-PCRs.

Some extraction methods used for isolation of RNA from plants (MacKenzie *et al.*, 1997; Rowhani *et al.*, 1995; Salzman *et al.*, 1999; Nassuth *et al.*, 2000) rely on chemicals such as PVP-40 and β-ME to overcome the effects of PCR inhibitors. These types of chemicals are applied in the phenol-chloroform method. Similar to MacKenzie *et al.*, (1997), the attempt to reduce the levels of inhibitors in this study with the addition of 2.5 % PVP-40 and 1 % β-ME to the QIAzol reagent, improved the number of positive QIAzol extracted samples considerably (from 40 % to 72 % for leaf punches; from 24 % to 60 % for mealybugs) (Fig. 3.4 a,b). This highlights and confirms the importance of an extraction technique that is efficient in removing PCR inhibitors. While the QIAzol method was improved, even the modified version remained unreliable, as reproducibility of replicated PCRs of the same samples remained relatively low (Table 3.2). Based on the nested RT-PCR results, the phenol-chloroform RNA extraction method was superior to the other RNA extraction methods tested.

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However, as the quality of the RNA is of great importance for real-time qRT-PCR, the extraction methods were compared for use with real-time RT-PCR. For real-time RT-PCR analysis of extraction methods, the Qiagen RNeasy® Plant Mini kit was included, because it has been successfully used for RNA isolation (MacKenzie *et al.*, 1997; Nassuth *et al.*, 2000; Osman *et al.*, 2007). The Qiagen RNeasy® Plant Mini kit has been the preferred method for RNA isolation for the detection of GLRaV-3 (Nassuth *et al.*, 2000; Osman *et al.*, 2007; Osman *et al.*, 2008) and the kit has proven to be more efficient in RNA isolation when compared to traditional methods (Osman *et al.*, 2007). The advantages of commercial kits lies in their fast, easy and a more gentle approach (no toxic chemicals) to RNA isolation (Lewis, 1997; Farrell, 2005). Furthermore, RNA isolation with commercial kits should yield good quality and pure RNA (Lewis, 1997; Read, 2001; Farrell, 2005). In comparison, the more traditional phenol-chloroform method has a number of disadvantages, e.g. toxicity and time required. Phenolic compounds absorb light in the UV range. Therefore, small amounts of phenol that remain in purified RNA can lead to an overestimate of the concentration of total RNA present in samples that are quantified with a UV spectrophotometer (Ginzinger, 2002). Furthermore, traces of phenol may cause a reduction in the efficiency of reverse transription (RT) (Ginzinger, 2002). This could explain the high total RNA concentration readings found in the QIAzol extractions, but poor PCR efficiency (Figs 3.5, 3.6), low number of positive samples (Fig. 3.4c,d) and low reproducibility (Table 3.2).

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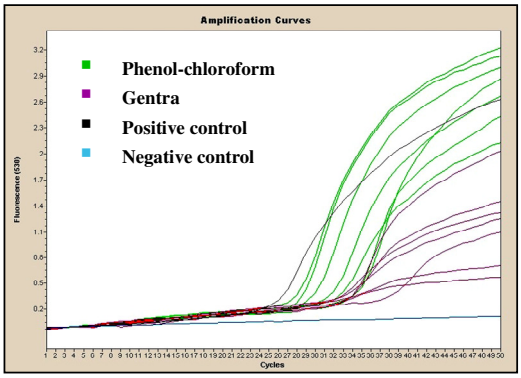
a)



b)

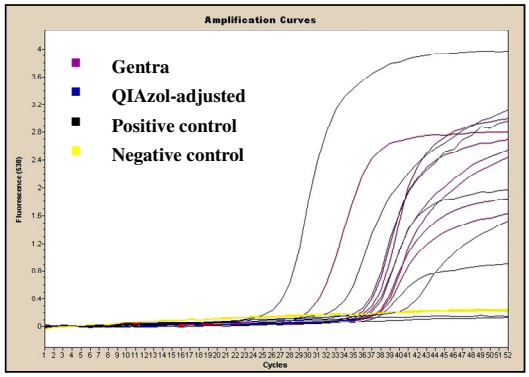


Fig. 3.5. Extraction efficiency comparisons from single leaf punch and single mealybug samples (n = 7 per extraction method) for a) phenol-chloroform and Gentra and b) Gentra control and QIAzol-adjusted.

When extraction efficiency comparisons between 1) the phenol-chloroform, Gentra and QIAzol-adjusted 2) the phenol-chloroform, QIAzol-adjusted and Qiagen RNeasy methods using standardized samples were done, the QIAzol-adjusted method gave contradictory results. Comparing the extraction efficiency using phenol-chloroform, Gentra and QIAzol-adjusted, the QIAzol-adjusted method appeared to be superior for extractions from mealybugs than plants (Fig. 3.6a,c). However, comparing extraction efficiency of phenol-chloroform, QIAzol-adjusted and Qiagen RNeasy, the QIAzol-adjusted method appeared to be a better method for extractions from plants than mealybugs (Fig. 3.6b,d). Real-time RT-PCR results consistently showed higher RT-PCR efficiencies with the phenol-chloroform method for both leaf punch and mealybug samples, compared to the other extraction methods (Figs 3.5, 3.6). Even though the Qiagen RNeasy mealybug extractions (Fig. 3.6d) appeared to have higher template concentrations than the phenol-chloroform mealybug extractions, the number of GLRaV-3 positive testing mealybug samples was 100 % for the phenol-chloroform samples, compared to 83 % for the Qiagen RNeasy method

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(Fig. 3.4d). The quality of the real-time RT-PCR products of all extraction methods was viewed under UV light. Non-specific amplification was observed with the Gentra, QIAzol-adjusted and Qiagen RNeasy extracted samples, but not the phenol-chloroform extracted samples. From the results of this study, it is clear that the phenol-chloroform method was the best RNA isolation method, producing higher quality RNA. Furthermore, the present study shows that the choice of extraction method should depend on the aim of a specific study.

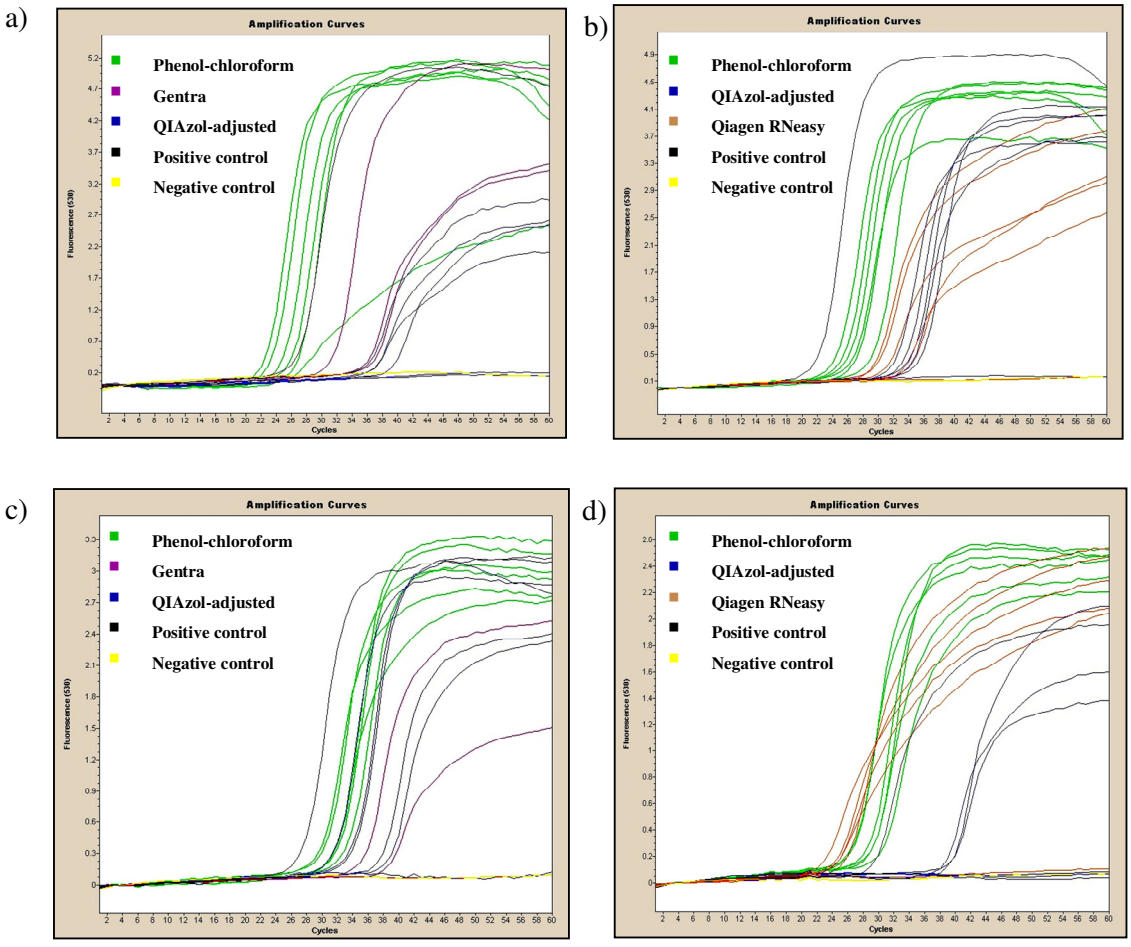

Fig. 3.6. Extraction efficiency from leaf (a, b) and mealybug samples (c, d), six replicates each for phenol-chloroform, QIAzol-adjusted and Qiagen and three replicates for Gentra.

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The study confirms that the extraction method affects the integrity and purity of extracted RNA from grapevine and mealybugs. Extraction methods should, therefore, be carefully selected for reliable and diagnostic use in real-time RT-PCR.

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CHAPTER FOUR

Development of a standard curve real-time quantitative RT-PCR for GLRaV-3 in grapevines and mealybugs (Hemiptera: Pseudococcidae)

Abstract

Template or nucleic acid concentration in a sample can be successfully determined with the use of standard curves and real-time RT-PCR. The accuracy of real-time quantitative RT-PCR (qRT-PCR) depends on the accuracy with which the standard curve was designed. The objective of this study was to develop a standard curve real-time qRT-PCR assay for quantifying *Grapevine leafroll-associated virus 3* (GLRaV-3) in grapevines and the grapevine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae). DNA and cRNA standards were developed for quantifying GLRaV-3 in grapevines and mealybugs. The DNA standards with a wider detection and amplification range were more sensitive and stable than the cRNA standards. However, the cRNA standard is subjected to reverse transcription (RT) with the experimental samples. Therefore, the cRNA standard provides information on the input RNA concentration prior to the RT step and can account for differences in efficiencies of cDNA synthesis. Both standards can be used for quantifying GLRaV-3 in grapevines and mealybugs. However, the purpose of the study and the accuracy required determines the choice of standard curve. The DNA standard model is best applied as a quantification method where initial RNA concentration is not relevant. For the purpose of this study, the standard curve using the cRNA standards is a better model for quantifying GLRaV-3 in grapevines and mealybugs.

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4.1 Introduction

The ability of real-time RT-PCR to detect and quantify PCR products as they accumulate throughout the PCR assay enables researchers to accurately determine initial concentration levels of target RNA in a given sample (Bustin, 2000; Fronhoffs *et al.*, 2002; Ginzinger, 2002; Balaji *et al.*, 2003; Gachon *et al.*, 2004; Wong & Medrano, 2005). Essentially, there are two main real-time quantification techniques, absolute and relative quantification (Bustin, 2000; Ginzinger, 2002; Pfaffl, 2003; Wong & Medrano, 2005). The type of quantification depends on the level of accuracy required. Absolute quantification is the most reliable quantification technique as it yields an absolute (accurate) calculation of target RNA concentration in unknown samples with the use of a standard curve (Bustin, 2000; Ginzinger, 2002; Pfaffl, 2003; Tichopád, 2004). In comparison, relative quantification uses housekeeping genes (internal standards) as reference genes and the expression level of a target gene (or DNA/RNA concentration in the case of a virus) in a sample is compared to that in the reference gene (Thellin *et al.*, 1999; Pfaffl, 2001; Ginzinger, 2002; Pfaffl *et al.*, 2002; Pfaffl, 2003). The comparison between the sample and reference gene only indicates whether the target gene is present in a higher or lower concentration in the sample than in the reference gene (Bustin, 2000).

To apply an absolute quantification technique, a standard curve is generated from a range of serially diluted PCR target template specific samples, known as external standards, which have defined concentrations (e.g. copy number or ng/µl) of the target nucleic acid (Ginzinger, 2002; Liss, 2002; Tichopád, 2004; Wilkening & Bader, 2004; Wong & Medrano, 2005). The range of serially diluted standards prepared from these, must fall within the expected concentration range of the target (Ginzinger, 2002; Wong & Medrano, 2005). For real-time qRT-PCR a standard curve is created by amplifying the external standard samples and then plotting the log

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concentrations (X-axis) of the standard samples against their crossing points (Y-axis) (Bustin, 2000; Rutledge & Côté, 2003). The crossing point (CP) or threshold cycle (C_t) of a sample is the cycle at which the target fluorescence rises above background fluorescence (Ginzinger, 2002; Bustin *et al.*, 2005). The CP depends on the initial target concentration; the lower the CP value of a sample the higher the starting target concentration in that sample (Ginzinger, 2002).

Because absolute quantification uses standard curves, the accuracy, stability and reproducibility of qRT-PCR, relies heavily on the standard curve design (Fronhoffs *et al.*, 2002; Pfaffl, 2003, 2004). Despite the accuracy with which the concentration of a standard is determined, the final results are still expressed relative to a unit of interest e.g., ng/µl, copies per reaction (Pfaffl, 2004). Real-time RT-PCR is highly sensitive and factors such as human error, e.g. pipetting error, quality of RNA extraction and RT efficiency can influence the quantification step (Farrel, 2005; Wong & Medrano, 2005). Even good laboratory practice cannot overcome these factors, especially not human errors, no matter how small their contribution (Farrell, 2005). For these reasons, the term absolute quantification is rather inaccurate and a more appropriate description is standard curve quantification (Ginzinger, 2002). The term absolute should be regarded as an actual concentration value obtained in a relative manner.

Different types of external standards exist, for example, recombinant RNA (recRNA), recombinant DNA (recDNA), dsDNA, ssDNA, cRNA, synthetic oligonucleotides and purified RT-PCR product (Bustin, 2000; Pfaffl & Hageleit, 2001; Ginzinger, 2002; Wong & Medrano, 2005). The choice of standard curve depends on the purpose of the experiment or study and the degree of accuracy required.

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The objective of this study was to develop a standard curve real-time qRT-PCR assay for quantifying *Grapevine leafroll-associated virus 3* (GLRaV-3) in grapevines and the grapevine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae).

4.2 Material and Methods

4.2.1 Sources of plants and insects

Leaf samples (leaf punches) were collected from GLRaV-3 infected (virus source) grapevine cv. Cabernet franc (CF) plants and stored at -70 ^{o}C. The CF plants were tested before experiments with nested RT-PCR using the external and internal primer sets and a protocol adapted from Ling *et al.* (2001) (Douglas & Krüger, 2008) to confirm their virus status.

Planococcus ficus was obtained from a non-viruliferous laboratory colony maintained at the University of Pretoria, South Africa. Mealybugs were given an acquisition access period (AAP) of 1-4 days on the CF plants. After the AAP the mealybugs were collected and stored at -70 ^{o}C. Sub-samples of *P. ficus* (n = 20) were tested for GLRaV-3 with nested RT-PCR (Ling *et al.*, 2001; Douglas & Krüger, 2008) prior to experiments to confirm their virus-free status. The identification of *P. ficus* was confirmed using the multiplex PCR assay developed by Saccaggi *et al.* (2008).

4.2.2 Samples and RNA extraction

Twelve leaf samples, that previously tested positive for GLRaV-3 (Chapter 3), were used for the design of the external standard curve. Each of the 12 samples consisted of six leaf punches. The six punches were taken along the main veins of a

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CF grapevine leaf. Two leaf punches per vein were placed into one of three collection tubes, equaling a total of six leaf punches per collection tube (Chapter 3). Single leaf punches and the mealybugs feeding on these leaf punches collected from CF plants, that previously tested positive for GLRaV-3 (Chapter 3), were used for absolute quantification and testing of the standard curve.

GLRaV-3 was extracted from leaf punches and mealybugs using the phenol-chloroform total RNA extraction protocol and standardization process discussed previously in Chapter 3;Appendix B.

4.2.3 Construction of external standards

The following techniques were adopted for the generation of the standard curve 1) cloning of real-time RT-PCR specific product, 2) purified real-time RT-PCR specific product and 3) *in vitro* transcription using real-time RT-PCR products as templates in order to generate nucleotide specific RNA.

4.2.3.1 Cloning

In order to design an external standard, cloning of the GLRaV-3 real-time RT-PCR product was attempted, to be followed by *in vitro* transcription. Two GLRaV-3 positive leaf punch samples were used for real-time RT-PCR. The real-time RT-PCR protocol discussed in Chapter 3;Appendix B was followed. The total 20 µl volume of each RT-PCR product was separated by a 1.5 % agarose gel (stained with EtBr) electrophoresis in 1 x TAE buffer. Both samples were gel-purified individually using the SV Wizard SV Gel and PCR Clean-up System (Promega, Cat # A9282) (Appendix C). The purified RT-PCR product was used for cloning with the pGEM®-T and pGEM®-T Easy Vector System (Promega, TM042) according to the

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manufacturer's specifications (for the protocol see Appendix C). To determine whether cloning was successful, the cloned product was sequenced.

4.2.3.2 Purified RT-PCR product

To design a DNA standard (DNA external standard), 2 µl of each of the 12 phenol-chloroform extracted leaf samples were used in real-time RT-PCR. The total volume of the amplified DNA of each of the 12 samples was divided into three volumes (7 µl, 7 µl and 6 µl, increasing the sample size to 36) and each of the 36 samples were separated by a 2 % agarose gel (stained with GoldView™ (SBS Genetech) nucleic acid stain) electrophoresis in 1 x TAE buffer. The total volume of amplified DNA was divided into three, to increase the sample number for gel-purification. All 36 samples were gel-purified individually using the Roche High Pure PCR product purification kit (Appendix C). After completing the purification, the 36 samples were pooled together and thoroughly homogenized. The samples were pooled together to have enough of the standardized DNA material for use in many real-time qRT-PCR tests. The DNA was quantified with a Nanodrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd) by averaging 20 replicated A_{260} absorbance determinations. Serial 10-fold dilutions of the external standard were made in nuclease-free water to obtain a DNA external standard range.

To generate the DNA standard curve, 2 µl of each of the DNA standards were used for real-time PCR. The 20 µl reaction contained 15.4 µl PCR grade water, 0.6 µl of the GLRaV-3 primer/probe mixture (discussed in Chapter 3) and 4 µl of the TaqMan® Master mix. For DNA templates the amplification reaction conditions included an incubation step of 95 ^{o}C for 10 min, followed by 60 cycles of 95 ^{o}C for 15s, 60 ^{o}C for 1 min and 72 ^{o}C for 1s. The LightCycler® software 4.0, using the automated method, generated the DNA standard curve. The DNA standard curve was

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saved as a separate file (external DNA standard curve). This file could later be imported into an absolute quantification run to determine concentration of unknown samples.

4.2.3.3 In vitro transcription

In order to design a cRNA standard (cRNA external standard) of the exact size and nucleotide sequence composition as the viral target RNA, the following protocol was used: 2 µl of each of the 12 phenol-chloroform extracted leaf samples were used in real-time RT-PCR. The real-time RT-PCR protocol described in Chapter 3 was followed. However, the 56 F forward primer, was modified (T7-56F) by incorporating a T7 promoter sequence at the 5' end, 5'-TAATACGACTCACTATAGGGAGAAAGTGCTCTAGTTAAGGTCAGGAGTGA -3' following Totzke *et al.* (1996); Fronhoffs *et al.* (2002) and Vijgen *et al.* (2005). A primer/probe mixture (T7-GLRaV-3 primer/probe mixture) containing 5 µl of the T7-56F (100 pmol/µl) and 5 µl of the 285 R (100 pmol/µl) primers and 1 µl of 181 P (100 pmol/µl) were added to 49 µl SABAX water to adjust the final volume to 60 µl, was made. The 22 µl reaction contained 14.75 µl PCR grade water, 0.6 µl of the T7-GLRaV-3 primer/probe mixture, 18 u HPRI RNase inhibitor, 40 u M-MLV reverse transcriptase, 4 µl of the TaqMan® Master mixture and 2 µl RNA extraction.

The total volume of the amplified DNA of each of the twelve samples were pooled together and thoroughly homogenized. The DNA was quantified with a Nanodrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd) by averaging 5 replicated A_{260} absorbance determinations.

A total of 92.8 µl (eight volumes of 11.6 µl each) of the RT-PCR product was used for *in vitro* transcription by the MAXIscript® protocol (Ambion, Cat # AM1308 - AM1326) using the manufacturer's specifications (Appendix C). The volume of the

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in vitro transcription products were pooled together (cRNA external standard) and thoroughly homogenized.

The cRNA standard was quantified with a Nanodrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd) by averaging 5 replicated A_{260} absorbance determinations. Serial 10-fold dilutions of the external standard were made in nuclease-free water to obtain a cRNA external standard range.

To generate the cRNA standard curve, 4 µl of each of the cRNA external standards were used for real-time RT-PCR (Chapter 3). The LightCycler® software 4.0, using the automated method, was used to generate the cRNA standard curve. The cRNA standard curve was saved as a separate file (external cRNA standard curve). This file could later be imported into an absolute quantification run to determine concentration of unknown samples.

4.2.4 Sensitivity and reproducibility of standard curves

Experiments were conducted to assess, sensitivity and reproducibility of the DNA and cRNA standard curves.

Conversion of nanogram (ng) to copies/µl was done following equation 1 (E1):

$$Amount[copiesl/\mu l] = \frac{6x10^{23}[copies/\mu l] \times concentration[g/\mu l]}{MW[g/mol]} \qquad \text{E1}$$

The molecular weight of dsDNA (DNA standard) and ssRNA (cRNA standard) is calculated by [(number of base pairs) x (660 daltons/base pair)] and [(number of bases) x (340 daltons/base)], respectively and 1 mol = 6 x 10^{23} molecules (copies) (Roche Applied Science, 2003).

To assess the intra-assay reproducibility of the DNA and cRNA standards, dilutions ranging from 1:10 to 1:10^5 (approximately 1.77 x 10^9 to 1.77 x 10^5 GLRaV-

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3 copies/µl) and 1 to 1:10^4 dilution range (approximately 5.822 x 10^{10} to 5.822 x 10^6 GLRaV-3 copies/µl) respectively, were analyzed in five replicates per run.

To assess the inter-assay (run-to-run) variation of both DNA and cRNA standards, dilutions of the DNA and cRNA standards, ranging from of 1:10 to 1:10^6 dilution range (approximately 1.77 x 10^9 to 1.77 x 10^4 GLRaV-3 copies/µl) and 1 to 1:10^4 dilution range (approximately 5.822 x 10^{10} to 5.822 x 10^6 GLRaV-3 copies/µl) respectively, were analyzed in five different experiments (PCR assays).

To minimize the effects of continuous freezing and thawing of the cRNA external standards, aliquots of each external standard dilution was made. The CP values of the aliquot cRNA standards were plotted against their log concentrations and compared to the external cRNA standard curve which was generated from the stock external standards.

4.2.5 Quantification of GLRaV-3 in grapevines and mealybugs

Quantification of GLRaV-3 in grapevines and mealybugs using the DNA standard curve, was performed by including one DNA standard in the qRT-PCR assay. After completion of the run, the external DNA standard curve file was imported into the run and the GLRaV-3 concentration in the grapevines and mealybugs was calculated from the external DNA standard curve.

Quantification of GLRaV-3 in grapevines and mealybugs using the cRNA standard curve was performed by 1) including one cRNA external standard in the qRT-PCR assay and importing the external cRNA standard curve file and by 2) including five cRNA standards (internal cRNA standard curve) in the qRT-PCR assay, from which a standard curve was generated after completion of the run and the GLRaV-3 concentration in the grapevines and mealybugs was calculated using this internal cRNA standard curve as reference.

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4.2.6 Real-time qRT-PCR

Real-time qRT-PCR was performed following the real-time RT-PCR protocol (Chapter 3) with 2 µl of extracted RNA or DNA standards or 4 µl cRNA standards. Real-time qRT-PCR was performed with a LightCycler® instrument (Roche Applied Science) and quantitative results were obtained with the LightCycler® software 4.0 (using the automated method).

4.3 Results

4.3.1 Construction of external standards

The attempt to clone the real-time RT-PCR product was not successful; the sequencing results only gave the vector sequence.

A DNA external standard generated from purified RT-PCR product, with a final concentration of 4.96 ng/µl pure specific DNA was obtained. To finally determine the GLRaV-3 viral load in grapevines and mealybugs a standard preparation of known concentration was subjected to serial 10-fold dilutions ranging from 1:10 to $1:10^{10}$ (approximately 1.77×10^{9} to 1.77 GLRaV-3 copies/µl) and was successfully amplified and generated a standard curve with an amplification efficiency (determined by the LightCycler® software) of 1.914 (Fig. 4.1a,b).

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a) b)

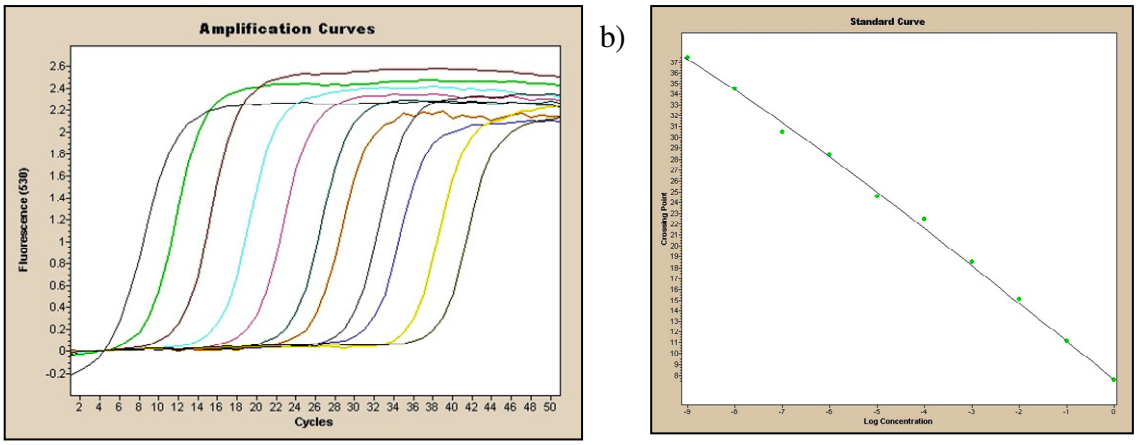

Fig. 4.1. Construction of DNA standard curve. LightCycler™-assisted real-time PCR of purified RT-PCR product a) serially diluted DNA external standards ranging from approximately 1.77 x 10^9 to 1.77 GLRaV-3 copies/µl. Plotting fluorescence against cycle number the crossing points (CP) were determined by the automated method provided by LightCycler™ software 4.0 and b) the CP values were plotted against the logarithm of the starting template concentration, showing a linear relationship.

In vitro transcription of RT-PCR products was successful and a cRNA external standard, with a concentration of 8.38 ng/µl was obtained. A serial 10-fold cRNA standard dilution range of 1:10 to 1:10^7 (approximately 5.822 x 10^9 to 5.822 x 10^3 GLRaV-3 copies/µl) was successfully amplified and used to generate a standard curve with an amplification efficiency (determined by the LightCycler® software) of 1.946 (Fig. 4.2 a,b).

As expected, the amplification plots of both DNA and cRNA standards shifted to the right with increasingly higher dilutions yielding increasingly higher CP values, as the starting template concentration became less (Figs 4.1a, 4.2 a).

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a) b)

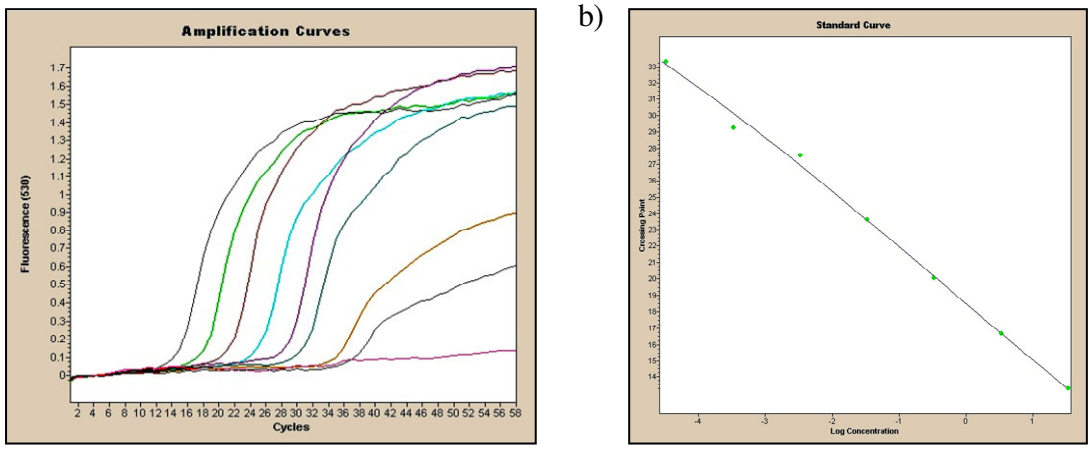

Fig. 4.2. Construction of cRNA standard curve. LightCycler™-assisted real-time RT-PCR of *in vitro* transcribed RT-PCR product a) serially diluted cRNA external standards ranging from approximately 5.822 x 10^9 to 5.822 x 10^3 GLRaV-3 copies/µl. Plotting fluorescence against cycle number the crossing points (CP) were determined by the automated method provided by LightCycler™ software 4.0 and b) the CP values were plotted against the logarithm of the starting template concentration, showing a linear relationship.

4.3.2 Sensitivity and reproducibility of standard curves

The mean CP, standard deviations (SD) and coefficient of variation (CV) for the intra-assay reproducibility (five replicates per run) (Table 4.1) and inter-assay (run-to-run) (five replicated experiments) (Table 4.2) reproducibility of both the DNA and cRNA standards were calculated.

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Table 4.1. Mean, standard deviation (SD) and coefficient of variation values for intra-assay variation of DNA and cRNA standards. An increase in variation is shown in DNA and cRNA standards with low template concentration.

		CP		
Samples	**copies/µl**	**Mean**	**SD**	**Coefficient of variation**
DNA standard	1.78×10^{9}	7.466	0.126	1.691
	1.78×10^{8}	11.616	0.102	0.881
	1.78×10^{7}	16.958	0.300	1.769
	1.78×10^{6}	22.028	0.169	0.769
	1.78×10^{5}	27.242	0.413	1.517
cRNA standard	5.822×10^{10}	14.084	0.122	0.865
	5.822×10^{9}	17.398	0.216	1.243
	5.822×10^{8}	21.978	0.594	2.704
	5.822×10^{7}	26.330	0.323	1.226
	5.822×10^{6}	30.894	0.444	1.436

Table 4.2. Mean, standard deviation (SD) and coefficient of variation values for inter-assay variation of DNA and cRNA standards. An increase in variation is shown in DNA and cRNA standards with low template concentration

		CP		
Samples	**copies/µl**	**Mean**	**SD**	**Coefficient of variation**
DNA standard	1.78×10^{9}	7.248	0.399	5.510
	1.78×10^{8}	11.058	0.253	2.287
	1.78×10^{7}	15.020	0.406	2.702
	1.78×10^{6}	18.854	0.479	2.539
	1.78×10^{5}	22.694	0.959	4.226
	1.78×10^{4}	24.656	0.514	2.086
cRNA standard	5.822×10^{10}	12.500	0.454	3.628
	5.822×10^{9}	15.828	0.473	2.988
	5.822×10^{8}	19.586	0.389	1.986
	5.822×10^{7}	23.736	0.597	2.514
	5.822×10^{6}	27.342	0.536	1.959
	5.822×10^{5}	29.226	0.377	1.292
	5.822×10^{4}	33.292	0.466	1.399

Variation in intra-assay reproducibility was small (Fig. 4.3), with low SD values and CV values (Table 4.1). However, an increase in variability, even though

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small, could still be detected in the standard samples with lower template concentration per reaction (Fig. 4.3). The inter-assay reproducibility of the DNA and cRNA standards showed increased variability in the external standard samples with lower template concentration per reaction (Fig. 4.4 a,b). For the cRNA standards 5.84 x 10^{22} to 5.84 x 10^{15} transcripts could be detected in two of the five assays, however the quantitative range was determined from 5.84 x 10^{22} to 5.84 x 10^{16}.

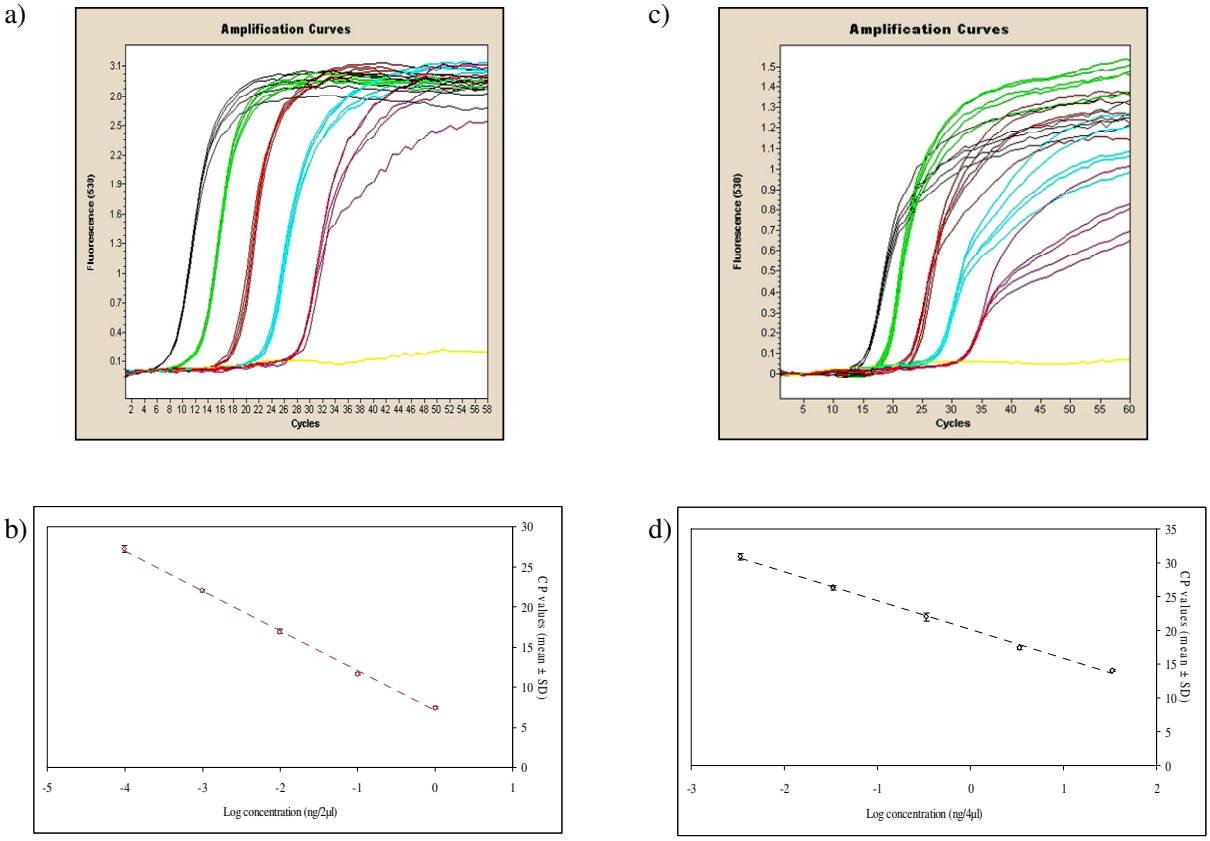

Fig. 4.3. Intra-assay reproducibility test of a, b) DNA and c, d) cRNA external standard models. Plotting fluorescence against cycle number, the CP values were determined by the automated method provided by LightCycler™ software 4.0. The mean CP and standard deviation values were plotted against the logarithm of the initial template concentration.

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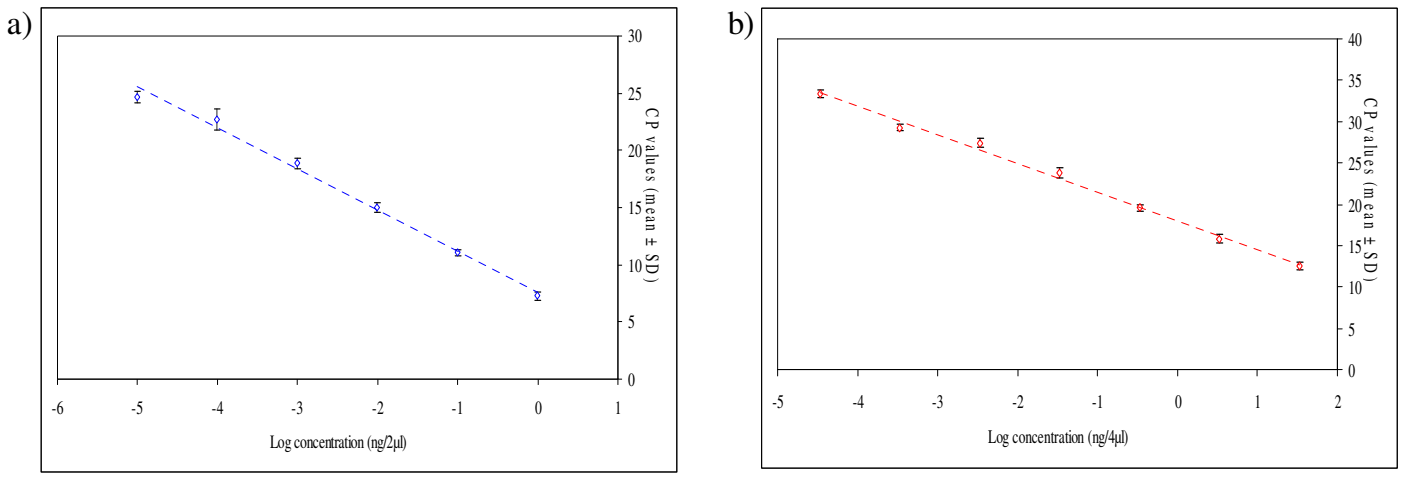

Fig. 4.4. Inter-assay reproducibility test of a) DNA and b) cRNA external standard model. The mean CP and standard deviation values of five external standard samples from five replicated experiments were plotted against the logarithm of the initial template concentration. An increase in variation is detected in the DNA and cRNA standards with lower template concentration.

To determine the reproducibility of the aliquoted, stored cRNA standards, the CP values were plotted against their log concentrations and compared to the external cRNA standard curve which was generated from the freshly-diluted stock cRNA standards. It appeared that continuous freezing and thawing (more than five freeze/thaw cycles) of the aliquot standards caused a shift in the standard curve (Fig. 4.5).

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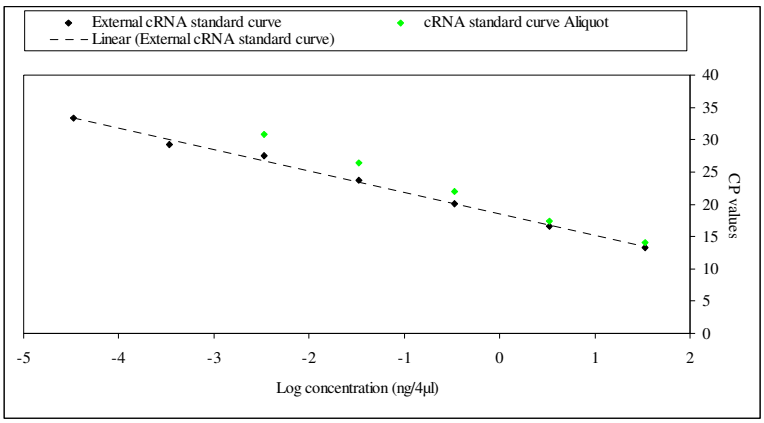

Fig. 4.5. Comparison of cRNA standard curve generated from aliquot external standard samples with external cRNA standard curve. The CP values were plotted against the logarithm of the initial template concentration. A shift (higher CP values) of the aliquot cRNA standard curve is present in the low template concentration external standard samples.

The first two standards (higher template concentration) showed little variation from the external cRNA standard curve. However, the variation increased in the more dilute standards (Fig. 4.5) as expected, due to increased variability observed in the inter- and intra-assay assessments. To further validate these results, the aliquot standards were assessed, together with the stock cRNA standards in a single qRT-PCR assay (Fig. 4.6). The last two cRNA standards (Fig. 4.6) showed a smaller shift in the standard curve as they had been subjected to less freeze/thaw cycles than the first five dilutions, thus illustrating the deleterious effect of freeze/thaw cycles on the nucleic acid template for PCR.

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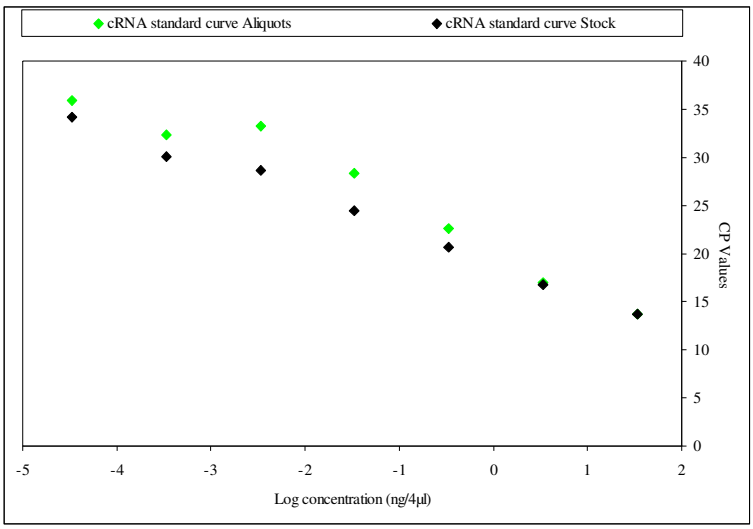

Fig. 4.6. Comparison of cRNA standard curve generated from aliquot external standard samples with cRNA standard curve generated from stock external standard samples. The CP values were plotted against the logarithm of the initial template concentration. A shift (higher CP values) of the aliquot cRNA standard curve is present in the low template concentration external standard samples.

4.3.3 Quantification of GLRaV-3 in grapevines and mealybugs

GLRaV-3 quantification results obtained from the DNA standard curve are shown in Fig. 4.7, Table 4.3.

The internal cRNA standard curve and GLRaV-3 concentrations in grapevines and mealybugs derived from the internal cRNA standard curve were compared to the external cRNA standard curve (Fig. 4.8b, Table 4.3). A shift in the standard curve was detected in the standards with lower template concentrations.

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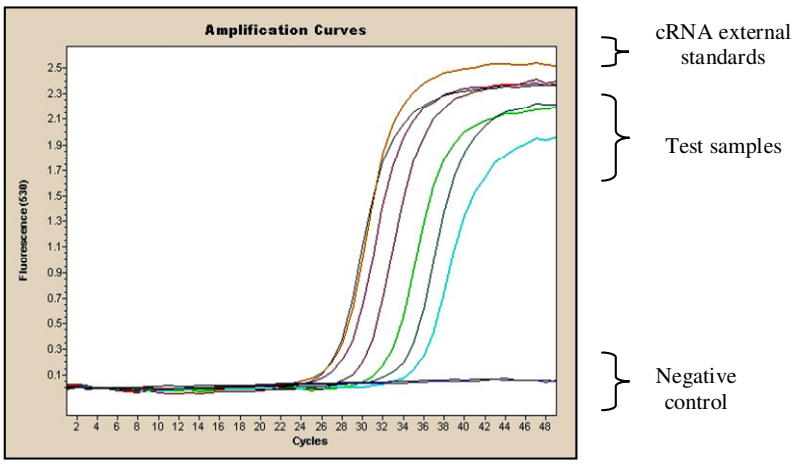
a)



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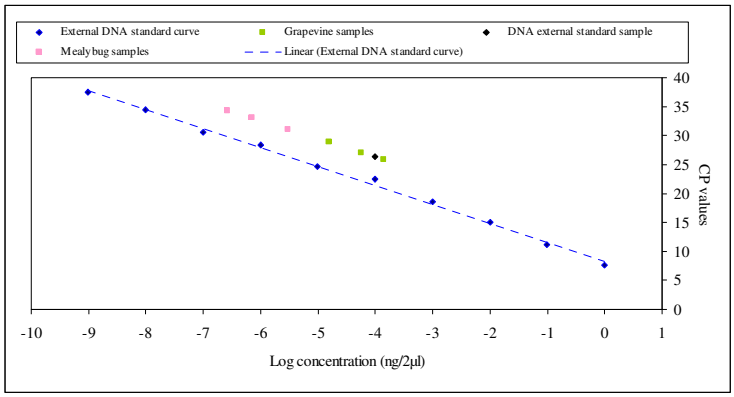

Fig. 4.7. Quantification of GLRaV-3 concentration in grapevine and mealybug samples with external DNA standard curve, a) LightCycler™-assisted real-time RT-PCR of external DNA standard and of six samples b) quantification of GLRaV-3 in samples was achieved by extrapolation of the CP values of the samples against the standard curves.

The LightCycler® enables users to select and de-select standards and only the standards that are selected are used for absolute quantitative analysis by the LightCycler® software 4.0. This enabled absolute quantitative analysis of GLRaV-3 concentration in grapevines and mealybugs by selecting only one cRNA standard in

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the qRT-PCR assay (excluding the remaining four standards) and importing the external cRNA standard curve after completion of the run. The GLRaV-3 concentration in the grapevines and mealybugs was calculated from the external cRNA standard curve (Fig. 4.8c, Table 4.3). When this quantitative analysis was performed, the grapevine and mealybug samples fit onto the external cRNA standard curve.

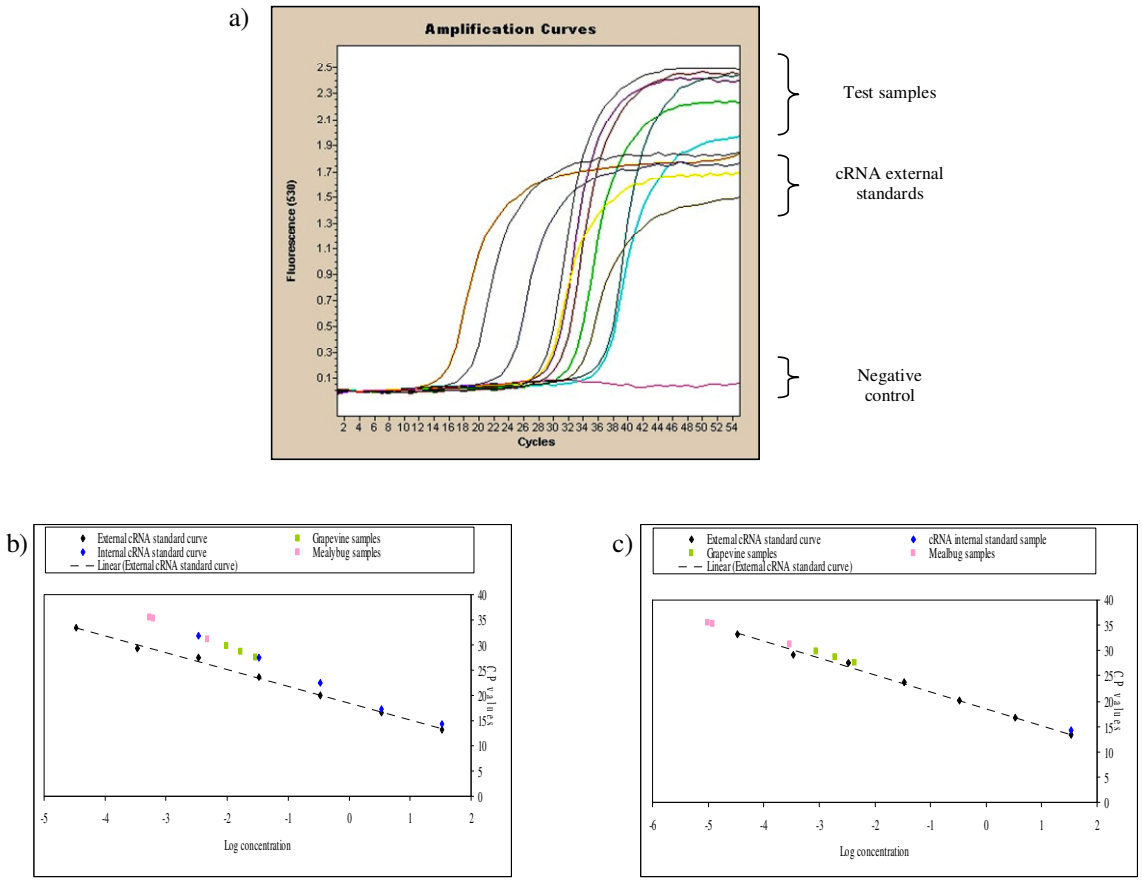

Fig. 4.8. Comparison between quantification of GLRaV-3 concentration in grapevine and mealybug samples using a, b) internal and a, c) external cRNA standard curves. a) LightCycler™-assisted real-time RT-PCR of serially diluted cRNA external standards ranging from approximately 5.822 x 10^{10} to 5.822 x 10^{6} GLRaV-3 copies/µl and of six samples. b, c) quantification of GLRaV-3 in samples was achieved by extrapolation of the CP values of the samples against the standard curves.

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The DNA and cRNA standards allowed quantification of GLRaV-3 in grapevine leaves and mealybugs. The GLRaV-3 concentration was found to be higher in grapevines than mealybugs when applying the DNA and cRNA standard curve quantification technique (Figs 4.7, 4.8, Table 4.3). The DNA and cRNA standard curve models gave different template concentration values for each unknown sample. However, if one method gave a higher template concentration for a particular sample compared to another sample, the second method showed a similar trend (Table 4.3).

Table 4.3. CP and GLRaV-3 concentration values of grapevine (L) and mealybug (M) samples from qPCR and qRT-PCR using the DNA standard curve and cRNA standard curve models, respectively. For the cRNA standard curve both internal and external cRNA standard curve methods were used.

	DNA Standard curve		cRNA standard curve		
Sample	CP value	Measured GLRaV-3 concentration	CP value	Measured GLRaV-3 concentration	
				Internal standard curve	External standard curve
L1	25.79	1.38E-04	27.57	2.93E-02	4.32E-03
M1	31.11	2.98E-06	31.22	4.68E-03	2.93E-04
L2	28.84	1.58E-05	29.76	9.75E-03	8.77E-04
M2	34.3	2.64E-07	35.31	6.06E-04	1.20E-05
L3	27.06	5.64E-05	28.69	1.67E-02	1.92E-03
M3	33.01	7.12E-07	35.54	5.40E-04	9.94E-06

4.4 Discussion

The use of recDNA, recRNA and cRNA external standards as quantification techniques has proven to be sensitive and highly reproducible (Pfaffl & Hageleit, 2001; Fronhoffs *et al.*, 2002; Olmos *et al.*, 2005; Vijgen *et al.*, 2005; Ruiz-Ruiz *et al.*, 2007; Bertolini *et al.*, 2008; Saponari *et al.*, 2008). In this study real-time RT-PCR product could not be cloned, possibly due to difficulties in transforming plasmids with the relatively small fragment size (254 bp). Therefore, the method of constructing the external standards using cloned RT-PCR products, followed by *in*

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vitro transcription (recRNA) was not possible. As an alternative method, purified RT-PCR product was used as an external standard for constructing a DNA standard curve for quantification.

The DNA standard proved to be highly sensitive enabling detection of the entire 10-fold serial dilution range of 1:10 to $1:10^{10}$ (approximately 1.77×10^9 to 1.77 GLRaV-3 copies/µl). The limitation of DNA standard curve methods is that they quantify only the starting cDNA template in unknown samples and do not provide any information on the amount of template prior to the RT step (Pfaffl, 2004). Inevitably, differences in cDNA synthesis efficiency are present and will affect the outcome of qRT-PCR results.

In view of this limitation, *in vitro* transcription was performed on real-time RT-PCR product for construction of a cRNA external standard (Fronhoffs *et al.*, 2002; Vijgen *et al.*, 2005). The cRNA standard was less sensitive than the DNA standard with detection of up to only the $1:10^7$ (approximately 5.822×10^9 to 5.822×10^3 GLRaV-3 copies/µl) 10-fold cRNA external standard dilution. However, the benefit of using a cRNA standard curve for quantification is that the cRNA standards are also subjected to the RT step along with the unknown samples (Pfaffl, 2004) and therefore help to account for the relative efficiency of this step.

Intra- and inter-assay variation evaluated the quantitative sensitivity and precision of the standard curves (Wong & Medrano, 2005). An estimate of standard curve reproducibility can be determined by analyzing the standard deviation (SD) of the CP values produced from replicated runs (Rutledge & Côté, 2003). In the present study, the standard deviation was relatively low in the intra- and inter-assay variation of both standards, indicating high reproducibility. However, an increase in variability, even though small, in the standard samples with lower template concentration per reaction in both assays was detected for the DNA and cRNA standards. This was

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expected as the intra- and inter-assay variability is generally higher in standards with a lower starting template concentration (Wong & Medrano, 2005).

RNA standard curve methods have shown more intra- and inter-assay variation than DNA standard curve methods (Pfaffl & Hageleit, 2001; Pfaffl, 2004). This was also observed in the present study, with slightly higher intra- assay variation in the cRNA standards as compared to the DNA standards. A possible explanation for this occurrence could be that RNA is more readily degraded by temperature changes (freezing and thawing) and enzymatic degradation than DNA (Pfaffl & Hageleit, 2001). Therefore, DNA is generally more stable than RNA (Pfaffl & Hageleit, 2001), which may be useful when designing standard curves. However, for quantification of viral RNA, such as GLRaV-3, RNA standards are a better option, becauss DNA standards do not account for the RT step or any efficiency differences that may be present within this step and may not give accurate quantification of the unknown samples (Pfaffl, 2004).

In the present study, the unstable nature of RNA was confirmed as increased variability was displayed in replicates of aliquots of the low template concentration cRNA external standards. The increased variability became more apparent with repeated use. The cRNA external standards with the highest template concentration appeared to be more stable. Aliquots of the cRNA standards were initially prepared in an attempt to reduce the risk of RNase degradation. Assessment of the different aliquots of any given dilution proved to be valuable, as repeated freezing and thawing caused a shift in the cRNA standard curve. However, it is best to make aliquots of the stock and preserve them at -70 ^{o}C. When aliquots of standards are made, results of this study suggest that regular evaluations of the standards by comparing the standard curve generated from the aliquots with that of the stock standards are essential. If a shift in the standard curve is detected, new aliquots should be made.

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The stability of RNA standards can be improved by diluting the specific RNA external standards into external non-PCR reacting sources of RNA (background) such as transfer RNA (tRNA) (Fronhoffs *et al.*, 2002; Pfaffl, 2004) or even healthy control samples, e.g. grapevine and mealybug total RNA extract. RNA is more easily degraded by RNases in the presence of water (Farrell, 2005) especially when present in low concentration (Pfaffl, 2004). Dilutions of PCR target-specific RNA made in PCR non-target RNA may reduce RNase activity on the PCR specific RNA template. In the current study the cRNA standards consist only of PCR template-specific RNA molecules (Pfaffl, 2004), viz. GLRaV-3. The cDNA synthesis efficiency of this may differ from that of grapevine and mealybug total RNA extraction samples which is a mixture of both PCR template specific RNA and non-target RNA. The use of only target specific RNA may lead to inaccurate quantification results. Performing the cRNA standard dilution range in background RNA may even correct for difference in the efficiencies of the RT step and increase the accuracy of quantification results (Pfaffl, 2004).

In addition, a RNA isolation method that yields high quality and pure RNA, such as the phenol-chloroform method (Chapter 3), together with standardization, in terms of input total RNA, may also lead to increased quantification accuracy. This is especially important when comparing viral concentration in different samples. To further correct for RT-PCR efficiency differences, an internal control that has a constant level of expression in a sample should be included (Pfaffl, 2004).

Quantification of GLRaV-3 in grapevine leaves and mealybug samples was performed using the DNA and cRNA external standards. Template concentration in the test samples can be calculated (automatically done by the LightCycler® software 4.0) by 1) including one external standard in a run and importing a previously designed external standard curve after completion of the run, or by 2) including five

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(Bustin, 2000) external standards in a run (internal standard curve). Even though the former analysis method has the advantage of 100 % reproducibility and no variation in the standard curve, the disadvantage is that it does not account for day-to-day differences such as pipetting error or differing RT efficiencies (Pfaffl, 2004). The second (latter) analysis may prove to be more accurate, because the unknown samples and standard samples are exposed to the same experimental conditions of a given PCR run. For the DNA standard the previously designed external DNA standard curve was imported for quantification analysis, because the DNA standard does not account for variation in the RT step. However, both methods were applied to quantification with the cRNA standard. The two quantification methods gave different template concentration values for each unknown sample. The choice between external or internal standard curve quantification should be based on the level of accuracy required, and each one has its own advantage.

As previously mentioned, the term absolute quantification is rather inaccurate. However, this term is commonly used by researchers (Pfaffl, 2004). This is acceptable, if it is understood that the absolute quantification is absolute in the sense that it provides an actual concentration value. Unlike, relative quantification that only gives a concentration of a gene in a sample as being more or less than the concentration of the gene in the reference (Bustin, 2000). However, it is extremely important to remember that the absolute quantification value was still obtained in a relative manner (Pfaffl, 2004) and that the readings are only an indication of template concentration and should not be accepted as the actual template concentration.

The DNA and cRNA standard models are highly sensitive, enabling quantification of GLRaV-3 in first-instar *P. ficus* nymphs. However, the choice of standard curve (DNA or cRNA) and quantification analysis (external or internal standard curve) determines the sensitivity and reproducibility of qRT-PCR (Pfaffl,

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2004) and should be carefully selected based on the purpose of the study. For example, the standards developed in the current study can be used for comparing GLRaV-3 concentration in mealybugs exposed to different feeding times. The DNA standard quantification method can be used in studies where the initial target RNA concentration is irrelevant to the purpose the study.

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CHAPTER FIVE

General discussion and conclusion

5.1 General discussion

The objective of this study was to develop a real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) assay for quantifying *Grapevine leafroll-associated virus 3* (GLRaV-3) in grapevine and mealybugs.

Real-time RT-PCR enables detection and quantification of PCR products as they accumulate throughout the PCR assay (Giulietti *et al.*, 2001; Wong & Medrano, 2005). The assay is a highly sensitive technique and allows accurate determination of initial concentration levels of target RNA in a given sample (Bustin, 2000; Fronhoffs *et al.*, 2002; Ginzinger, 2002; Balaji *et al.*, 2003; Gachon *et al.*, 2004; Wong & Medrano, 2005). Two quantification techniques are available, absolute and relative quantification (Bustin, 2000; Ginzinger, 2002; Pfaffl, 2003; Wong & Medrano, 2005).

The advantage of using absolute quantification is that this technique allows accurate calculation of target RNA concentration in unknown samples. The disadvantage is the effort in developing a standard curve (Giulietti *et al.*, 2001; Wong & Medrano, 2005). In comparison, relative quantification only gives arbitrary values of target RNA concentration in unknown samples (Bustin, 2000; Wong & Medrano, 2005). Therefore, absolute quantification is a more accurate method for quantifying target RNA concentration in a sample and has been adopted in the current study.

There are however, certain assumptions and limitations when applying absolute quantification. Firstly, that amplification efficiency of sample and standards are assumed to be the same (Souazé *et al.*, 1996). Secondly, the concentration range of standards falls within the expected concentration range of target RNA in the experimental samples (Ginzinger, 2002; Wong & Medrano, 2005). Thirdly, the

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accuracy, stability and reproducibility of absolute quantification depends on the accuracy of the standard curve design (Fronhoffs *et al.*, 2002; Pfaffl, 2003, 2004). Furthermore, sample-to-sample (day-to-day) variation, when quantifying target RNA concentration in different individuals, may be present due to differences in RNA integrity, purity and quantity as well as experimental procedures (Wong & Medrano, 2005). This may lead to differences in reverse transcription (RT) and may give inaccurate quantification results. The selection of a standard is very important for accurate absolute quantification. Different types of standards exist, for example, recombinant RNA (recRNA), recombinant DNA (recDNA), dsDNA, ssDNA, cRNA, synthetic oligonucleotides and purified RT-PCR product (Bustin, 2000; Pfaffl & Hageleit, 2001; Ginzinger, 2002; Wong & Medrano, 2005). DNA standards have the advantage of being more stable than RNA standards (Pfaffl & Hageleit, 2001; Pfaffl, 2004). However, the stability of RNA can be improved by diluting the specific RNA standards into external non-PCR reacting sources of RNA (background RNA) such as transfer RNA (tRNA) (Fronhoffs *et al.*, 2002; Pfaffl, 2004). Furthermore, DNA standards have proven to be more sensitive, having a wider quantification range and are more reproducible than RNA standards (Pfaffl & Hageleit, 2001). The disadvantage of the DNA standards is that they are not subjected to the RT step (Pfaffl, 2004) and target RNA quantification is based on the starting cDNA concentration and not the initial RNA concentration. A DNA standard can be applied as a quantification method where initial RNA concentration is not relevant (Giulietti *et al.*, 2001). The choice of standard depends entirely on the purpose of the experiment or study and the degree of accuracy required.

In the present study, a DNA and cRNA standard model was developed for quantifying GLRaV-3 in grapevines and mealybugs. The DNA standard model was more sensitive and had a wider detection range than the cRNA standard model,

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confirming findings by Pfaffl & Hageleit (2001). In the present study the unstable state of the cRNA standard model was reflected in higher intra- and inter-assay variation than DNA standard model and variability became more apparent with repeated use. This reflected the importance of diluting the specific RNA standards into external non-PCR reacting sources of RNA (background) such as transfer RNA (tRNA) (Fronhoffs *et al.*, 2002; Pfaffl, 2004), to improve stability.

The standard curve quantification methods developed in this study can be improved by adding additional control measures. This include a no template control, which accounts for DNA contamination (Wong & Medrano, 2005). Further, differences in RT can be corrected for with the use of internal controls (normalizers) (Wong & Medrano, 2005). The internal control should be RNA that is consistently expressed in a sample (e.g. plant or insect). Any RT differences will be reflected in the target and internal control RNA (Ferre, 1992). The use of commercial kits (e.g. LightCycler® TaqMan® Master kit (Roche Applied Science)) can minimize day-to-day variation (Phillips, 2004). The quality of the purified RNA is critical for successful and reliable use in real-time qRT-PCR (Bustin, 2000; Pfaffl, 2003; Gachon *et al.*, 2004). The majority of RNA extractions are contaminated with low levels of DNA and protein that interfere with the efficiency of RT-PCR (Pfaffl, 2004). Therefore, the RNA isolation method used should provide good quality and pure RNA. Standardizing the amount of starting material may further improve accuracy of qRT-PCR.

Four different RNA extraction methods (phenol-chloroform, Gentra Purescript® RNA Isolation kit, Qiagen QIAzol™ and Qiagen RNeasy® Plant Mini kit) using GLRaV-3 positive plant material and mealybugs were compared for real-time qRT-PCR. Sampling methods were standardized. The input total RNA was standardized at 50 ng. Selection of a RNA isolation method was based on the quality

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and purity of extracted RNA. Impurities in the extracted RNA samples were detected in the PCR amplification efficiency of real-time RT-PCR of three of the RNA isolation methods tested. A commercial PCR reagent kit (LightCycler® TaqMan® Master kit) was used for real-time RT-PCR. This aspect of the study produced a RNA isolation method that yielded high quality and pure RNA and the RNA isolation method was applied for the design of a standard curve.

Quantification experiments in the present study showed the DNA and cRNA standard models to be highly sensitive, enabling quantification of GLRaV-3 in first-instar *Planococcus ficus* nymphs. Even though the two methods gave different template concentration values for each unknown sample, both standard models showed a similar trend, e.g. if one method gave a higher template concentration for a particular sample compared to another sample, the second method yielded similar results.

The choice of standard curve (DNA or cRNA) determines the stability, reproducibility and accuracy of qRT-PCR (Pfaffl, 2004) and should be carefully selected based on the purpose of the study. DNA standards are best applied as quantification methods where initial RNA concentration is not relevant.

5.2 Conclusion

This study aimed to develop a real-time qRT-PCR assay to quantify GLRaV-3 in grapevine and mealybugs. In order to accomplish this, RNA isolation methods were tested for use in real-time qRT-PCR and two standard curve models were designed for absolute quantification.

The real-time qRT-PCR developed in this study includes a RNA isolation method that yields good quality and pure RNA. The real-time qRT-PCR assay itself consists of two standard curve models. Both models can be used to quantify GLRaV-

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3 in grapevines and mealybugs. However, the model of choice depends on the purpose of the study and the accuracy required. The principles for development of this technique can be applied to other virus-vector related studies.

Absolute real-time qRT-PCR can be successfully applied if the limitations are fully understood and the assumptions are addressed. It is also important to understand that the absolute quantification value is obtained in a relative manner (Pfaffl, 2004) and that the readings are only an indication of template concentration and should not be accepted as the actual template concentration. Therefore, the interpretation of quantitative data is very important.

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APPENDIX A

STANDARD BUFFERS, CHEMICALS AND MEDIUMS

A.1. RNA extraction buffer

NaCl (MW = 58.5)	0.1 M (0.585 g)
SDS (MW = 288.38)	2% (2 g)
Tris/HCl (pH9) (MW = 157.56)	50 mM (0.788 g)
EDTA (MW = 292.25)	10 mM (0.292 g)
β-mercaptoethanol (β-ME)	20 mM (140 µl)

Add 80 % distilled water. Add Tris/HCl and adjust pH to 9. Add remaining chemicals and make up to 100 ml with distilled water.

A.2. DEPC water

1 ml DEPC per 1000 ml deionized water

Deionized water	200 ml
DEPC	200 µl

Add DEPC in fume hood, loosen cap slightly and shake incubate overnight at 37 ^{o}C.

Autoclave for 20 min.

A.3. NaOH (10 M)

NaOH (MW = 40)	40 g

Make up to a 100 ml with distilled water.

A.4. QIAzol, β-mercaptoethanol and PVP-40

QIAzol	100 ml

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β-ME)	1 %
PVP-40	2.5 %

To dissolve the PVP-40, heat the mixture, but do not let the contents boil.

A.5. Wetting medium

NaOH (MW = 40)	50 mM (0.02 g)
EDTA (MW = 292.25)	2.5 mM (0.073 g)

Make up to 100 ml with distilled water.

A.6. 1 x GES buffer

Glycine NaOH (pH 9)	0.1 M
NaCl (MW = 58.5)	50 mM
EDTA (MW = 292.25)	1 mM
β-ME	1 %
Triton X-100	0.5 %

Add 80 % distilled water. Add glycine and adjust pH to 9 using NaOH. Add remaining chemicals and make up to 100 ml with distilled water. To dissolve the EDTA, heat buffer, but do not let the contents boil.

A.7. GLRaV-3 primer/probe mixture

56 F (100pmol/µl)	20 µl
285 R (100pmol/µl	20 µl
181 P (100pmol/µl	4 µl
SABAX water	196 µl

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A8. T7-GLRaV-3 primer/probe mixture

T7-56F (100pmol/µl)	5 µl
285 R (100pmol/µl)	5 µl
181 P (100pmol/µl)	1 µl
SABAX	49 µl

A.9. 50 x TAE

Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (2 mole)	242 g
Glacial acetic acid (100% acetic acid) (57.19ml = 1 mole)	57.1 ml
0.5 M Na_2 EDTA (pH 8.0)	100ml

Make up to 1000ml with distilled water

To prepare 0.5 M Na2 EDTA (pH8.0) add 186.1 g of disodium ethylenediaminetretraacetate x $2H_2O$ to 800ml of H_2O. Stir vigorously. Adjust the pH to 8.0 with NaOH (ca. 20g of NaOH). Sterilize by autoclaving. Hint: the disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ca. 8.0 by the addition of NaOH.

A.10. 1 x TAE buffer

50 x TAE	20ml
Distilled water	980ml

A.11. Ethidium bromide

Add 5 µl EtBr (10 mg/ml) per 200 ml agarose gel.

A.12. GoldView™ (SBS Genetech)

Add 10 µl GoldView per 200 ml agarose gel.

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A.13. 1.5 % Agarose gel

Agarose	3g
1 x TAE	200ml

A.14. 2 % Agarose gel

Agarose	4g
1 x TAE	200ml

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APPENDIX B

NUCLEIC ACID EXTRACTIONS AND PCR PROTOCOLS

B.1. Standardization

All working surfaces were cleaned with 10 M NaOH.

The initial steps in sample preparation for each extraction method were standardized. For each extraction method leaf punches and their associated mealybug nymphs were taken from -70 ^{o}C and immediately placed in liquid nitrogen, to avoid thawing of the samples. The samples were ground with pestles, whilst kept in liquid nitrogen. The samples were placed on ice and ground further. Thereafter, the first reagent for each protocol assessed was added to the samples and the samples were homogenized.

Samples were homogenized for 1 min using a Soniprep 150 Ultrasonic disintegrator (Optolabor (Pty) Ltd). Between each 20 seconds of the 1 min, there was a 10 second interval. The samples were further homogenized by aspirating them 20 times through a P20 pipette.

After the completion of each extraction protocol, each sample was re-suspended in 14 µl diethyl pyrocarbonate (DEPC) treated water, the samples were flash frozen with liquid nitrogen and stored at -70 ^{o}C for later analysis using RT-PCR.

The total RNA concentration of every extracted sample was measured on a Biowave S2100 UV/Vis Diode Array Spectrophotometer (Biochrom Ltd) or a Nanodrop ND-1000 Spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd). If the total RNA concentration of a sample was more than 50 ng/µl the sample was diluted to 25 ng/µl and 2 µl (50 ng) was used for RT-PCR. If the total RNA concentration of a sample was less than 50 ng/µl, a calculation was done to determine

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the volume of sample to be used for RT-PCR, to ensure that the final sample added to the PCR mixture contained 50 ng RNA.

B.2. Phenol-chloroform method

Samples were ground in 100 µl RNA extraction buffer. To the homogenized samples, 100 µl of phenol (purum, >= 96.0 % (calc. on dry substance, T)) was added and vortexed for 1 min. A 100 µl of chloroform was added to the samples and the samples were briefly vortexed. The samples were centrifuged at 4 ^{o}C for 10 min at 8000 x g. The top aqueous RNA layer was placed into a new tube. This step is known as a phenol-chloroform step.

An additional phenol-chloroform step was performed on each sample and thereafter 200 µl of chloroform was added to each sample. The samples were vortexed for 1 min and centrifuged at 4 ^{o}C for 10 min at 8000 x g. The upper aqueous layer of each sample was taken and 30 µl of 3 M NaOAc (pH 5.2) and 900 µl of cold 100 % EtOH were added to the samples and mixed well. The samples were placed at –80 ^{o}C for 1 h for precipitation. The pellets were spun down at 4 ^{o}C for 30 min at 8000 x g. The supernatants were poured off and each pellet washed with 100 µl of 70 % EtOH and centrifuged at 4 ^{o}C for 10 min at 8000 x g. The EtOH was discarded and each pellet left to air dry for 10 min. Finally the pellets were dissolved in 14 µl DEPC treated water, flash frozen with liquid nitrogen and stored at -70 ^{o}C for later analysis using nested and real-time RT-PCR.

B.3. Gentra Purescript® RNA Isolation

Samples were ground with pestles in 300 µl Cell Lysis Solution. For protein-DNA precipitation 100 µl of Protein-DNA Precipitation Solution was added to each homogenized cell lysate and inverted 10 times. The samples were placed into an ice

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bath for 5 min. The samples were then centrifuged for 3 min at 16000 x g and the precipitated proteins and DNA formed a tight pellet.

For RNA precipitation the supernatants containing the RNA (leaving behind the precipitated protein-DNA pellet) were poured into clean 1.5 ml microfuge tubes containing 300 µl 100 % Isopropanol (2-propanol) and the samples were mixed by gently inverting them 50 times. The samples were centrifuged at 16000 x g for 3 min; the RNA was visible as a small, translucent pellet. The supernatants were poured off and the tubes drained on clean absorbent paper. To the samples 300 µl 70 % EtOH was added and inverted 50 times to wash the RNA pellet. The samples were centrifuged at 16000 x g for 1 min, where after the EtOH was carefully poured off. The tubes containing the samples were inverted and each tube drained on clean absorbent paper. The samples were left to air dry for 15 min. To elute, 14 µl of DEPC treated water was added to each sample and the samples were placed on ice for 30 min. The samples were vortexed for 5 s and centrifuged, flash frozen with liquid nitrogen and stored at -70 ^{o}C for later analysis using nested and real-time RT-PCR.

B.4. Qiagen QIAzol™ RNA isolation

Samples were ground with pestles in 100 µl QIAzol reagent each. The homogenized samples were centrifuged at 4 ^{o}C for 10 min at 12000 x g. The supernatants were placed into new tubes and left at room temperature for 5 min. Thereafter, 20 µl of chloroform (0.2 ml per 1 ml QIAzol) was added to the samples. The samples were vortexed for 1 min and centrifuged at 4 ^{o}C for 15 min at 12000 x g. The upper aqueous RNA phase was transferred to a new tube, 50 µl of Isopropanol was added to the samples and the samples were briefly vortexed. The samples were left at room temperature for 10 min and centrifuged at 4 ^{o}C for 10 min at 12000 x g. The supernatants were discarded and 100 µl of 75 % EtOH were added to the pellets

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and the pellets were centrifuged at 4 °C for 5 min at 7500 x g. The supernatants were discarded and the pellets left to air dry. The RNA was re-dissolved in 14 µl of DEPC treated water, flash frozen with liquid nitrogen and stored at -70 °C for later analysis using nested and real-time RT-PCR.

For the QIAzol-adjusted method the above protocol was followed, with one exception; 1 % β-mercaptoethanol (β-ME) and 2.5 % PVP-40 was added to the QIAzol reagent before proceeding with the extraction method.

B.5. Qiagen RNeasy® Plant Mini kit

Samples were ground with pestles in 450 µl buffer RLT (containing 10 µl β-mercaptoethanol per 1 ml buffer RLT). The homogenized samples were transferred to QIAshredders through aspirating and centrifuged for 2 min at maximum speed. The supernatants were transferred to new tubes and 225 µl of 100 % EtOH was added to each sample. Thereafter 650 µl of each sample was applied to RNeasy mini columns (pink column). The samples were centrifuged for 15 s at 8000 x g. The flow-through was discarded and 700 µl RW1 was added to each sample. The samples were centrifuged for 15 s at 8000 x g and 500 µl RPE (containing 4 volumes 100 % EtOH) was added to each sample. The samples were centrifuged for 15 s at 8000 x g and again 500 µl RPE was added to each sample. The samples were centrifuged for 2 min at 8000 x g and the flow-through of the samples was discarded. The samples were centrifuged for 1 min at full speed and thereafter placed in new collection tubes and 14 µl DEPC treated water was added to each sample. The samples were centrifuged for 1 min at 8000 x g and flash-frozen with liquid nitrogen and stored at -70 °C for later analysis using nested and real-time RT-PCR.

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B.6. Nested RT-PCR

The method described by La Notte *et al.* (1997) was also used for GLRaV-3 extraction. Briefly, plant sap from single petioles was spotted and single mealybug nymphs were crushed on a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) soaked in a 50 mM NaOH and 2.5 mM EDTA solution, then allowed to dry at room temperature for at least 1 hour. A 4 mm^2 piece of the spotted membrane was cut out and placed in 30 µl GES buffer (0.1 M glycine, pH 9, 50 mM NaCl, 1 mM EDTA, 1% β-mercaptoethanol, 0.5 % Triton X-100). The sample was then incubated at 95 ºC for 10 min and cooled on ice before being used for PCR.

The external sense primer 5'-TGTGGACAGCAATCTTCCAA-3' and anti-sense primer 5'-ACCAACTTCTCGGCGATCTC-3', which result in a 648 bp fragment, and the internal sense primer 5'-ATTTAGAAAGTACGATCGTGC-3' and anti-sense primer 5'-CAGTCAGGGGTAACATCTTA-3', which produce a 219 bp fragment, were used for GLRaV-3 detection (Ling *et al.*, 2001).

PCR was performed following the protocol adapted from Ling *et al.* (2001) by Marika van der Merwe (ARC-PPRI, South Africa). The 50 µl reaction volume contained 2.5 µl 2 % Triton-X, NH_4 buffer (final concentration: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01 % Tween-20), 1.5 mM $MgCl_2$, 10 mM DTT, 0.5 µM of each external primer, 175 µM of each dNTP, 18 u HPRI RNAse inhibitor, 40 u M-MLV reverse transcriptase, and 0.5 u BIOTAQ™ DNA Polymerase (BioLine, Luckenwalde, Germany) and 50 ng of the extraction. PCR thermal cycling conditions for the first-round PCR consisted of cDNA synthesis at 37 ºC for 45 min followed by denaturisation at 94 ºC for 2 min, then 35 cycles with denaturation at 94 ºC for 60 s, annealing at 50 ºC for 60 s and extension at 72 ºC for 2 min, with a final extension at 72 ºC for 10 min.

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Second-round amplification was carried out with 0.5 µl of the first-round product added to 50 µl of reaction mixture containing NH_4 buffer (as above), 1.5 mM $MgCl_2$, 0.5 µM of each external primer, 175 µM of each dNTP, and 0.5 u BIOTAQ™ DNA Polymerase (BioLine, Luckenwalde, Germany). The cycling program conditions were: 94 °C for 2 min, then 33 cycles at 94 °C for 60 s, 50 °C for 60 s and 72 °C for 2 min, and a final extension at 72 ºC for 10 min.

PCRs were performed using a 2720 Thermal Cycler (Applied Biosystems) thermocyclers. PCR products were visualized under UV light on a 1.5 % agarose gel stained with EtBr.

B.7. Real-time RT-PCR

Real-time RT-PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler® instrument (Roche Applied Science) and the protocol adapted from Osman & Rowhani (2006) and Osman *et al.* (2007). The 56 F (5'-AAGTGCTCTAGTTAAGGTCAGGAGTGA-3'), 285 R (5'-GTATTGGACTACCTTTCGGGAAAAT-3') and 181 P (5'-Fam-CAGGTAATAGCGGACTGAGACTGGTGGACA-BHQ-1-3') primers and probe designed by Osman & Rowhani (2006), which resulted in a 254 bp fragment, were used. The reaction contained 12.75 µl water (PCR grade), 0.6 µl of the GLRaV-3 primer/probe mixture, 18 u HPRI RNase inhibitor, 40 u M-MLV reverse transcriptase, 4 µl of the TaqMan® Master Mix and 0.5 – 4 µl (50 ng) of extractions. For RNA templates the reverse transcription and amplification reaction conditions were 45 °C for 35 min, 95 °C for 10 min, followed by 60 cycles of 95 °C for 15s, 60 °C for 1 min and 72 °C for 1s.

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B.8. Real-time RT-PCR (Modified 56 F primer)

The real-time RT-PCR protocol was followed. However, the 56 F forward primer, was modified (T7-56F) with a T7 promoter sequence at the 5' end, 5'-TAATACGACTCACTATAGGGAGAAAGTGCTCTAGTTAAGGTCAGGAGTGA-3'. The 22 µl reaction contained 14.75 µl PCR grade water, 0.6 µl of the T7-GLRaV-3 primer/probe mixture, 18 u HPRI RNase inhibitor, 40 u M-MLV reverse transcriptase, 4 µl of the TaqMan® Master mix and 2 µl RNA extraction.

B.9. Real-time PCR

Real-time PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler® instrument (Roche Applied Science). The 20 µl reaction contained 15.4 µl PCR grade water, 0.6 µl of the GLRaV-3 primer/probe mixture and 4 µl of the TaqMan® Master mix. For DNA templates the amplification reaction conditions included an incubation step of 95 ^{o}C for 10 min, followed by 60 cycles of 95 ^{o}C for 15s, 60 ^{o}C for 1 min and 72 ^{o}C for 1s.

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APPENDIX C

PCR product purification, cloning and *in vitro* transcription

C.1. SV Wizard SV Gel and PCR Clean-up System (Promega, Cat # A9282)

The desired band was cut out using an ethanol-cleaned scalpel blade and placed in a pre-weighed 1.5 ml tube. The weight of the gel slice was determined. To each 10 mg gel slice, 10 µl of membrane binding solution was added. To resuspend the gel slice in the membrane binding solution, the samples were briefly vortexed and centrifuged before incubation at 50-65 °C for 10 min. The samples were transferred to minicolumns and were incubated at room temperature for 10 min. Samples were centrifuged for 1 min at 10 000 x g and the contents of the collection tubes were discarded. This step was repeated. To the samples 700 µl membrane wash solution was added and the samples were centrifuged for 1 min at 10 000 x g. The collection tubes were emptied and 500 µl of membrane wash solution was added to each sample. The samples were centrifuged for 5 min at 10 000 x g and the collection tubes were emptied. The minicolumns were transferred to clean 1.5 ml tubes and 30 µl of nuclease-free water was added to each sample. The samples were incubated at room temperature for 1 min and centrifuged for 1 min at 10 000 x g.

C.2. Roche High Pure PCR product purification

C1.2.1. Gel Slice Purification

The desired band was cut out using an ethanol-cleaned scalpel blade and placed in a pre-weighed 1.5 ml tube. The weight of the gel slice was determined. To each 100 mg gel slice, 300 µl binding buffer was added. To resuspend the gel slice in the binding buffer, the samples were vortexed for 15-30 s. The samples

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were incubated for 10 min at 56 °C and vortexed briefly every 2-3 min. Once the gel slices were completely dissolved, 150 µl Isopropanol for every 100 mg gel slice was added and each sample was thoroughly vortexed. The entire contents of the 1.5 ml tube were aspirated into a filter tube (placed in a collection tube). If the volume exceeded 700 µl the volume was split and placed into more than one filter tube. The samples were centrifuged for 1 min at 13 000 rpm. The flow-through was discarded and the collection tubes were reconnected to their filter tube. Thereafter 500 µl of wash buffer was added to each sample and the samples were centrifuged for 1 min at 13 000 rpm. The flow-through was discarded. The collection tubes were reconnected to their filter tubes and 200 µl of wash buffer was added to each sample. The samples were centrifuged for 1 min at 13 000 rpm. The flow-through and collection tubes were discarded. The filter tubes were connected to 1.5 ml tubes and 50 µl SABAX water was added to each filter tube. The samples (closed) were left at room temperature for 10 min. The samples were centrifuged at full speed (14 000 rpm) for 1 min and the filter tube was discarded. The purified PCR product was in the 1.5 ml tube.

C1.2.2. PCR Product Purification

The volume of the PCR products was made up to 100 µl with SABAX water. The samples were transferred to 1.5 ml tubes and 500 µl binding buffer was added to each sample and the samples were vortexed. The entire contents of the 1.5 ml tube were aspirated into a filter tube (placed in a collection tube). If the volume exceeded 700 µl the volume was split and placed into more than one filter tube. The samples were centrifuged for 1 min at 13 000 rpm. The flow-through was discarded and the collection tubes were reconnected to their filter tube. Thereafter 500 µl of wash buffer was added to each sample and the samples were centrifuged

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for 1 min at 13 000 rpm. The flow-through was discarded. The collection tubes were reconnected to their filter tubes and 200 µl of wash buffer was added to each sample. The samples were centrifuged for 1 min at 13 000 rpm. The flow-through and collection tubes were discarded. The filter tubes were connected to 1.5 ml tubes and 50 µl SABAX water was added to each filter tube. The samples (closed) were left at room temperature for 10 min. The samples were centrifuged at full speed for 1 min and the filter tube was discarded. The purified PCR product was in the 1.5 ml tube.

C.3. pGEM®-T and pGEM®-T Easy Vector cloning (Promega, TM042)

Two GLRaV-3 positive leaf punch samples were used for real-time RT-PCR. The total 20 µl volume of each RT-PCR product was separated by a 1.5 % agarose gel (stained with EtBr) electrophoresis in 1 x TAE buffer. Both samples were gel-purified individually using the SV Wizard SV Gel and PCR Clean-up System (Promega, Cat # A9282). The standard Ligation reactions contained 5 µl 2 x Rapid Ligation Buffer T7 DNA Ligase, 1 µl pGEM®-T vector (50 ng), 2 µl or 3 µl of PCR products, 1 µl T4 DNA Ligase (3 Weiss units/µl) and deionized water to a final volume of 10 µl. The PCR product volume added was determined by the following equation:

$$ng\,of\,insert = \frac{ng\,of\,vector \times kb\,sizeof\,insert}{kb\,sizeof\,vector} \times insert\,to\,vector\,molar\,ratio$$

The insert:vector molar ratio was 3:1. The Positive Control Ligation reaction contained 5 µl 2 x Rapid Ligation Buffer T4 DNA Ligase, 1 µl pGEM®-T (50 ng), 2 µl Control Insert DNA, 1 µl T4 DNA Ligase (3 Weiss units/µl) and deionized water to a final volume of 10 µl.

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For transformation, two LB/ampicillin/IPTG/X-Gal plates each for each ligation reaction and two plates for determining transformation efficiency were prepared by adding 100 µl IPTG (100mM) and 20 µl of X-Gal (50 mg/ml) spread over the surface of plates and allowing absorption for 30 min at 37 °C.

The tubes containing the ligation reactions were briefly centrifuged and 2 µl of each ligation was added (on ice) to a sterile 1.5ml tube. For determining transformation efficiency of competent cells, 10 ng of uncut plasmid (PUC 18) was added to each 1.5 ml tube. Tubes containing frozen JM109 High Efficiency Competent Cells were put on ice for 5 min. The cells were mixed by gentle flicking and 50 µl cells were added to the ligation tubes and 100 µl to the Control PUC 18 (for determination of transformation efficiency). The tubes were gently mixed and placed on ice for 20 min. The cells were heat-shocked for 45-50 s in a water bath at 42 °C. Thereafter, the tubes were placed on ice for 2 min and 950 µl SOC medium (50 µl glucose and 50 µl Mg per 5 ml SOC) was added to each tube. The tubes were incubated with shaking for 1.5 h at 37 °C and 300 µl of each tube (transformation culture) was plated onto duplicate plates. For the transformation efficiency control a 1:10 dilution with SOC medium was plated onto duplicate plates. The plates were incubated overnight at 37 °C.

A total of 10 colonies were picked and cultured overnight in 3-5 ml SOC broth containing 50 µg/ml ampicillin. For plasmid extraction, 1.5 ml of the overnight culture was centrifuged at 4 °C for 1 min at 13200 rpm. The supernatant was poured off and the cells resuspended in 100 µl ice cold Solution 1. The cells were vortexed, kept at room temperature for 5 min and placed on ice for 5 min. Thereafter, 200 µl of Solution 2 (980 µl water, 20 µl NaOH mix, 100 µl (2 %) SDS) and the samples were mixed by inverting and incubated on ice for 5 min. To the samples 150 µl ice cold ammonium acetate (Solution 3) was added and mixed by vortexing. The samples

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were placed on ice for 15 min and centrifuged at 4 °C for 15 min at 13200 rpm. The supernatants were transferred to a new tube and 2 volumes 95 % EtOH was added and the samples incubated at -20 °C for 30 min. The samples were spun down for 15 min and the DNA pellet aspirated. The DNA pellet was washed with 70 % EtOH and centrifuged at 4 °C for 2 min at 13200 rpm. The DNA pellet was air dried and resuspended in 30 µl TE buffer and stored at -20 °C. To determine cloning success the samples were sequenced.

C.4. MAXIscript® *In vitro* transcription (Ambion, Cat # AM1308 - AM1326)

A total 92.8 µl (eight volumes of 11.6 µl each) of the real-time RT-PCR product was used for *in vitro* transcription (Ambion MAXIscript® kit), performed at 37 ^{o}C for 2 h in a 100 µl reaction mixture containing 20 µl Transcription Optimised 5X buffer, 10 µl DTT (100 mM), 2.5 µl (100 units) RNase inhibitor, 20 µl rNTP (2.5 µM) mix, 5 µg (11.6 µl) Linearized DNA template, 2µl (40 units) T7 RNA polymerse and molecular grade water added for a final volume of 100 µl.

DNase treatment of the *in vitro* transcription product was performed at 37 ^{o}C for 30 min in a reaction mixture containing 80 µl RNA in water, 12.5 µl RQ1 DNase 10 x reaction buffer and 12.5 µl RQ1 DNase. After incubation 12.5 µl of RQ1 DNase stop solution was added to terminate the reaction and the samples were incubated at 65 ^{o}C for 10 min to inactivate the DNase.

The total volume of each of the eight samples was divided into six volumes of 20 µl each, increasing the sample number to 48. Removal of free rNTP's from the *in vitro* transcription product was performed at -20 ^{o}C for 30 min (chilling step) in a reaction mixture containing 20 µl of DNase treated transcription reaction product, 30 µl molecular grade water and 5 µl 5M ammonium acetate. Each reaction mixture was vortexed and 165 µl (3 volumes) of 100 % EtOH added. After completion of the

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chilling step the samples were centrifuged for 15 min at maximum speed, the supernatant was discarded and the pellet washed with 70 % EtOH. The pellet was resuspended in 30 µl molecular grade water and the total volume of each of the 48 samples were pooled together.

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APPENDIX D

Primer and probe sequences

C1.1 NESTED RT-PCR

External sense primer	5'-TGTGGACAGCAATCTTCCAA-3'
Anti-sense primer	5'-ACCAACTTCTCGGCGATCTC-3'
Internal sense primer	5'-ATTTAGAAAGTACGATCGTGC-3'
Anti-sense primer	5'-CAGTCAGGGGTAACATCTTA-3'

C2.1 REAL-TIME RT-PCR AND REAL-TIME PCR

56 F	5'-AAGTGCTCTAGTTAAGGTCAGGAGTGA-3'
285 R	5'-GTATTGGACTACCTTTCGGGAAAAT-3'
181 P	5'-Fam-CAGGTAATAGCGGACTGAGACTGGTGGACA-BHQ-1-3'

C3.1 Modified 56 F primer

T7-56F	5'-TAATACGACTCACTATAGGGAGAAAGTGCTCTAGTTAAGGTCAGGAGTGA-3'

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LIST OF ABBREVIATIONS

A	Absorbance
µl	Microliters
µM	Micromolar
AAP	Acquisition access period
AMV-RT	*Avian myeloblastosis virus* reverse transcriptase
ARC	Agricultural Research Council
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CF	Cabernet franc
CRI	Citrus Research International
CsCl	Cesium chloride
CsTFA	Cesium trifluoroacetate
CP	Crossing point
cRNA	*In vitro* transcribed RNA
C_t	Cycle threshold
DEPC	Diethyl pyrocarbonate
CV	Coefficients of variation
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
DWV	*Deformed wing virus*
EDTA	Ethylenediaminetetraacetic acid

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EtBr	Ethidium bromide
EtOH	Ethanol
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLD	Grapevine leafroll disease
GLRaV-3	*Grapevine leafroll-associated virus 3*
GSTM1	Glutathione *S*-transferase 1
GTC	Guanidine thiocyanate
HybProbe	Hybridization Probes
IAP	Inoculation access period
ME	Mercaptoethanol
Min	Minutes
M	Molar
mM	Millimolar
MMLV-RT	*Moloney murine leukemia virus* reverse transcriptase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
ng/µl	Nanogram per microliter
PCR	Polymerase chain reaction
P. ficus	*Planococcus ficus*
PLRV	*Potato leafroll virus*
PMTV	*Potato mop top virus*
PPRI	Plant Protection Research Institute
PVA	*Potato virus A*

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PVP	Polyvinylpyrrolidone
PVX	*Potato virus X*
PVY	*Potato virus Y*
recDNA	Recombinant DNA
recRNA	Recombinant RNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
rNTP	Ribosomal nucleotide triphosphate
rpm	Revolutions per min
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SABAX	Sterile nonpyrogenic water
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
ssDNA	Single stranded DNA
ssRNA	Single stranded RNA
TAT	Turn around time
THRIP	Technology and Human Resources for Industry Programme
T_m	Melting temperature
Tris/HCl	Tris/Hydrogen chloride
tRNA	Transfer RNA
TRV	*Tobacco rattle virus*

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TSWV	*Tomato spotted wilt virus*
UBQ	Ubiquitin
UV	Ultraviolet
vol/vol	Ration of volume added to volume
qRT-PCR	quantitative RT-PCR

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