Industry allocated project number

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Indicate (X) client(s) to whom this final report is submitted. Replace any of these with other relevant clients if required.

FINAL REPORT
2014

Programme & Project Leader Information

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Project Information

Research Organisation Project number	PPRI Gos 4
Project title	The investigation into the reliability and sensitivity of RT nested - PCR, based on degenerate primers for the simultaneous detection of viruses of the Closterovirus, Ampelovirus, Foveavirus, and Vitivirus genera.

Fruit kind(s)	Grapevines		
Start date (mm/yyyy)	January 2012	**End date** January 2014	

Project keywords	RT nested-PCR; Closteroviridae, Flexiviridae; detection

Approved by Research Organisation Programme leader (tick box) [X]

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

Executive Summary

Give an executive summary of the total project.

The reliability of RT nested-PCR technique designed for the simultaneous detection of viruses associated with leafroll (LR) and rugose wood (RW) diseases (Dovas and Katis, J. Vir. Meth. 109, 217-226, 2003) was investigated. Results of an analysis of the virus status of multivirus-infected grapevines of various cultivars proved that the technique has the capability to efficiently and specifically amplify all known and yet unknown viruses associated with these two economically important diseases.

The technique was applied to the comparative analysis of viruses of grapevines cv. Shiraz with different Shiraz disease (SD) statuses. The study has led to the discovery of a new, highly divergent variant of Grapevine leafroll-associated virus 3 (GLRaV-3), and a new genetic variant of GVA. The results were published in the international journals.

A broad spectrum, two-step RT-PCR technique based on a single primer with random hexanucleotide sequence was investigated. This technique is potentially able to amplify all grapevine infecting viruses. The replicative form of dsRNA of viruses was targeted for RT-PCR amplification. Results revealed that, in contrast to the virus-infected herbaceous host, *N. benthamiana*, the isolation of RF from grapevines is difficult and impractical. Also, the amplification of total dsRNA using this technique could not find an application for the routine diagnosis of virus infections owing to the fact that plant RNA sequences contaminated dsRNA samples, and were efficiently amplified. The technique, however, can be applied to the analysis of viruses of grapevines that exhibit virus-like symptoms, but which are negative for all known viruses.

Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

According to recent data, grapevine is host to 60 viruses. The major concerns of grapevine industries world-wide are viruses of the *Closteroviridae* and *Betaflexiviridae* families, which include viruses associated with leafroll (LR) and rugose wood (RW) diseases. These diseases decrease productivity and longevity of vineyards, interfere with graft take, and negatively influence the quality of fruits and, ultimately, the quality of produced wines. Virus-infected grapevine can be cured by the application of virus elimination procedures, using heat treatment and in vitro tip cultures. However, the method is not 100% effective, and not all viruses are equally easy to eliminate. The propagation of virus-infected grapevines is then a possibility. Currently, the South African grapevine industry is using ELISA for simultaneous detection of GLRaV-1, -2 and -3. Although the technique is easy to use, it has limited sensitivity when compared with RT-PCR. In addition, it detects only three out of more than 10 viruses of the *Closteroviridae* family associated with leafroll disease (LR), and it does not detect viruses of the *Betaflexiviridae* family associated with rugose wood (RW) diseases. To ensure strict control of the the *Closteroviridae* and *Betaflexiviridae*-free status of grapevine propagative material, a sensitive virus detection technique that detects a broad range of viruses of these two families, is required. In 2003, Dovas and Katis (J. Vir. Meth. 109, 217-226, 2003) reported RT nested-PCR technique that can

simultaneously amplify all known members of *Closteroviridae* and *Betaflexiviridae* families. The technique is based on degenerate primers that overcome molecular differences between virus species, as well as molecular variability within a single species of virus. Thus, it can also amplify yet unknown members of these two virus families. The reliability of this technique was investigated in this study. Also, a broad spectrum two-step RT-PCR technique based on a single primer with random hexanucleotide sequence was investigated. This technique is potentially able to amplify all grapevine infecting viruses.

Workplan (materials and methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

Viruses of the families *Closteroviridae* and *Betaflexiviridae*, which can be RT nested-PCR amplified using the technique of Dovas and Katis (2003), and which have been investigated in this study are listed in Table 1.

Table 1.

Virus	Taxonomic classification (Family, Genus)
Grapevine leafroll-associated virus – 1 (GLRaV-1)	*Closteroviridae, Ampelovirus* (subgroup 1)
Grapevine leafroll-associated virus – 2 (GLRaV-2)	*Closteroviridae, Closterovirus*
Grapevine leafroll-associated virus – 3 (GLRaV-3)	*Closteroviridae, Ampelovirus* (subgroup 1)
Grapevine leafroll-associated virus - 4[a] (GLRaV-4)	*Closteroviridae, Ampelovirus* (subgroup 2)
Grapevine leafroll-associated virus – 5 (GLRaV-5)	*Closteroviridae, Ampelovirus* (subgroup 2)
Grapevine leafroll-associated virus – 6 (GLRaV-6)	*Closteroviridae, Ampelovirus* (subgroup 2)
Grapevine leafroll-associated virus – 9 (GLRaV-9)	*Closteroviridae, Ampelovirus* (subgroup 2)
Grapevine leafroll-associated virus – 10 (GLRaV-10)	*Closteroviridae, Ampelovirus* (subgroup 2)
Grapevine leafroll-associated virus – 11 (GLRaV-11)	*Closteroviridae, Ampelovirus* (subgroup 2)
Grapevine virus A (GVA)	*Betaflexiviridae, Vitivirus*
Grapevine virus B (GVB)	*Betaflexiviridae, Vitivirus*
Grapevine virus D (GVD)	*Betaflexiviridae, Vitivirus*
Grapevine virus E (GVE)	*Betaflexiviridae, Vitivirus*
Grapevine rupestris stem pitting -associated virus (GRSPaV)	*Betaflexiviridae, Foveavirus*

[a]Highlighted are Ampeloviruses of subgroup 2 which, according to recent molecular analysis, should be regarded as divergent genetic variants of the first discovered Ampelovirus, GLRaV-4 (Martelli et al. J.Pl.Path. 94, 7-19,2012). All members of this group are RT nested-PCR amplified using degenerate primers designed by Maliogka et al.(J. Virol. Meth. 154, 41-47, 2008). The technique was also used in this study (see Fig. 1).

Eight multiple virus-infected grapevine sources from PPRI collection which are shown in Table 2 were used to investigate the reliability of the technique.

Table 2.

Cultivar (code)	Virus status[a]	Viruses detected in this study[d]
Waltham Cross (WX)	GLRaV-1,-2,-3,-5, GVA, GRSPaV[b]	GLRaV-1,-2,-3,-10, GVA GRSPaV,
Pinot Noir (PN)	GLRaV-1,-2,-3, GFkV	NA
Chasselas (CH)	GLRaV-2,-6	GLRaV-2,-3,-5, GVB, GRSPaV
Barlinka (BAR)	GLRaV-4, GFkV	GLRaV-3, -4, GVA, GRSPaV
Ohanez (OH)	GLRaV-5, GFkV	GLRaV-3, -6 (-11), GVA, GVB
Black Spanish (BS)	GLRaV-1,-2,-3,-4,-5, GVA, GFkV	GLRaV-1,-2,-3,-4, GVA, unknown Flexivirus
Shiraz (SH)	GLRaV-2	GLRaV-3,-4
LN33 (LN)[c]	GLRaV-2, GVB	GLRaV-2,-3

[a] Determined using IEM (GGF Kasdorf, PPRI), except grapevine cv. Waltham Cross and LN33.
[b]See Proser, et al. (Vir. Res. 124, 151-159, 2007).
[c]The grapevine is strongly CB-affected. A severe variant of GLRaV-2 was mechanically transmitted to *N. benthamiana* (Goszczynski et al. J.Phytopath 144, 581-583, 1996). The plant was also GVB positive in RT-PCR (Goszczynski, not published).
[d]Grapevines LN33 and Shiraz were not analysed for Flexiviruses

In the study, cane cuttings of four grapevines from Vititec's nucleus collection (kindly supplied by Tobie Oosthuizen) were also used. The grapevines are shown in Fig. 1B as numbers 1, 2, 3 and 4, respectively.

RT nested-PCR technique for the simultaneous amplification of viruses of the genera *Foveavirus, Vitivirus* and *Closterovirus* of the *Betaflexiviridae* and *Closterovirdae* families reported by Dovas and Katis (2003) was modified by carrying out reverse transcription (RT), and two rounds of PCR separately. The amplification of DNA from members of these two families was also carried out separately. In all these amplifications the degenerate primers designed by Dovas and Katis (2003) were used.

Results and discussion

State results obtained and list any industry benefits. If applicable, include a short discussion covering ALL accumulated results from the start of the project. Limit it to essential information only.

THE APPLICATION OF THE TECHNIQUE TO VIRUS STATUS ANALYSIS OF MULTIPLE VIRUS-INFECTED GRAPEVINES OF VARIOUS CULTIVARS.

Strong RT nested-PCR amplification was obtained for all multiple virus-infected grapevines used in this study (Fig.1). In most cases, direct SSCP analysis of PCR amplicons showed a multiple band profile, which indicated multiple virus infection (Fig. 1). Results revealed that nucleus plants, marked in Fig. 1B as 1, 2, 3 and 4, were free of viruses of the *Closteroviridae* family but infected with a member of the *Betaflexiviridae* family, most probably *Grapevine rupestris stem pitting associated virus* (GRSPaV) (see discussion below).

Fig.1 A.

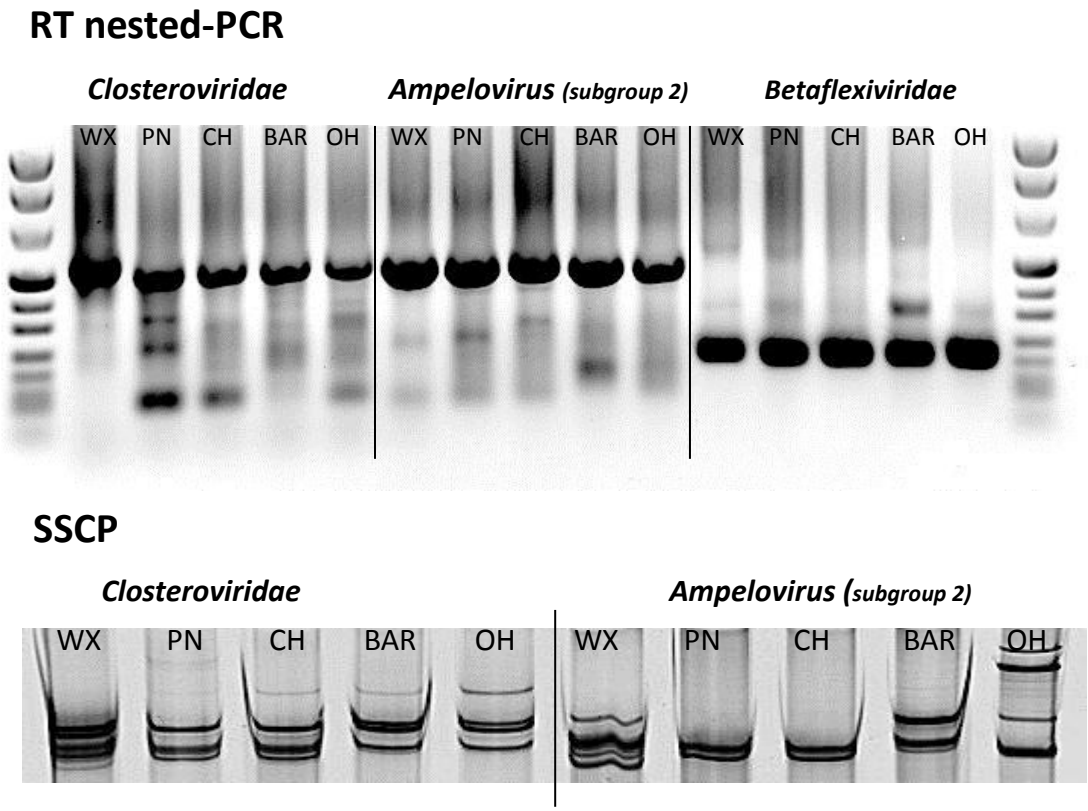
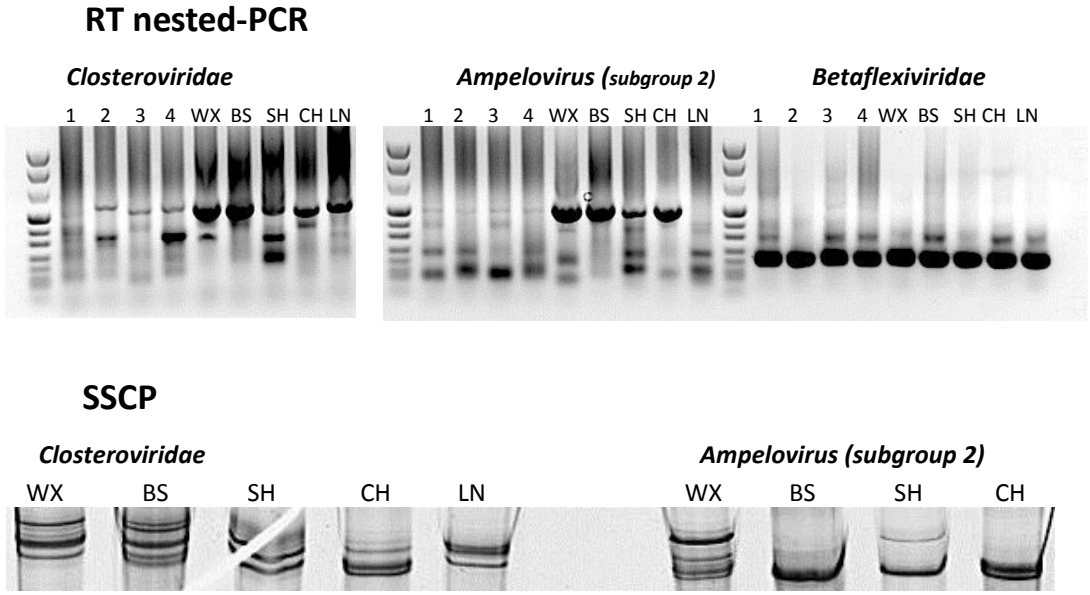


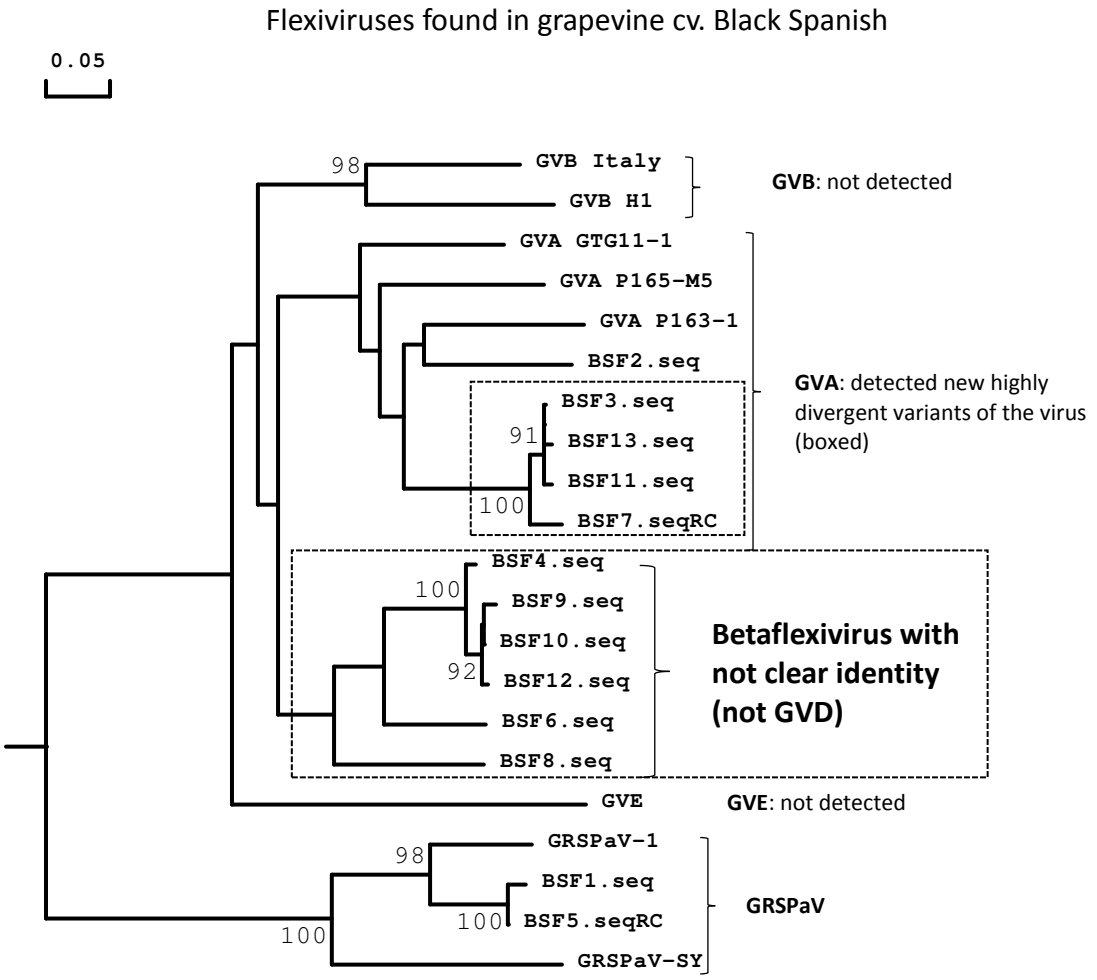
Fig. 1B.



Cloning and sequencing revealed the presence of all known viruses of the family *Closteroviridae* and *Betaflexiviridae*, except *Grapevine virus D* (GVD), *Grapevine virus E* (GVE), and the recently reported *Grapevine virus F* (GVF) (Table 2). The technique is virus-specific since no plant RNA sequences were found in the amplicons.

The example of the reliability of the technique to simultaneously amplify different members of *Betaflexiviridae* family as well as highly divergent genetic variants of these viruses are results of virus analysis of the multi virus-infected grapevine cv. Black Spanish (Fig. 2). The results showed that the grapevine is infected with populations of various divergent variants of GVA and GRSPaV. Analysis of the sequence data revealed a new, highly divergent variant of GVA. Also, the data strongly suggest that this grapevine is infected with an unknown member of the *Vitivirus* genus, closely related to GVA and GVB. An attempt was made to isolate this virus in *Nicotiana benthamiana*, but was unsuccessful.

Fig. 2.

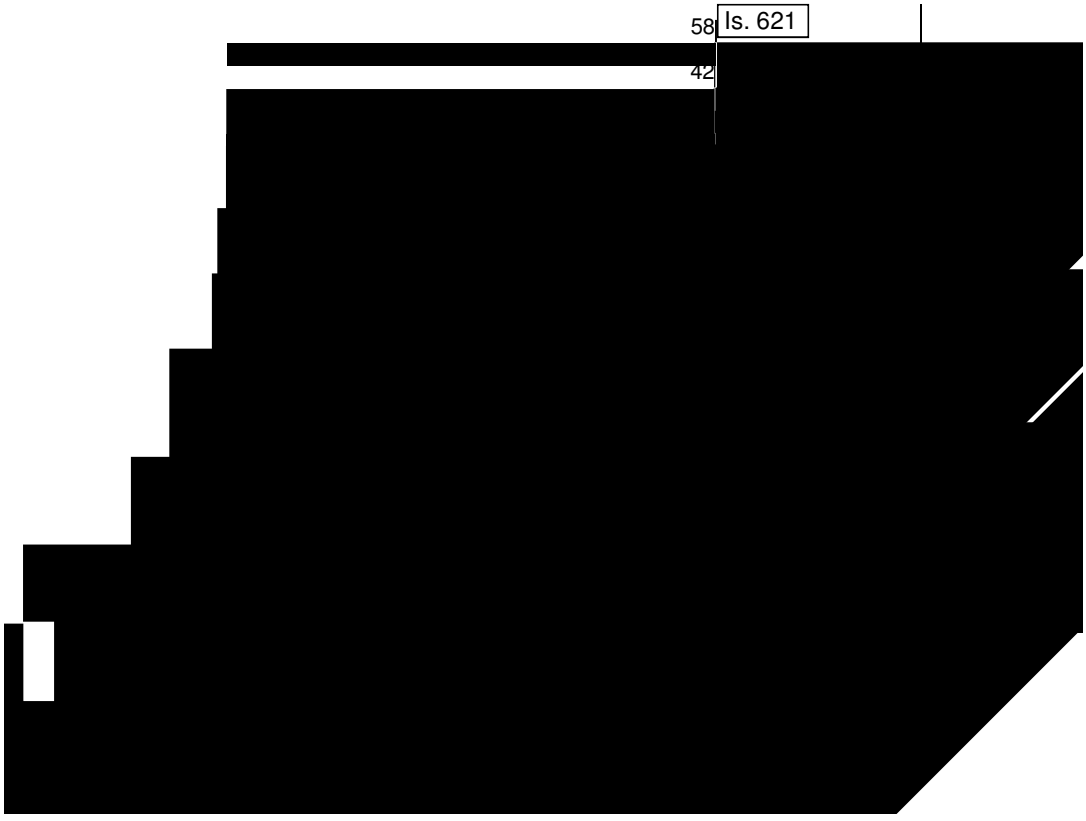


Results proved that the RT nested-PCR technique of Dovas and Katis (2003) allows precise analysis of genetic heterogeneity of members of the *Betaflexiviridae* and *Closteroviridae* families. The technique was applied to analysis of these viruses in grapevines cv. Shiraz with different Shiraz disease (SD) statuses. The studies led to the discoveries of a new highly divergent variant of GLRaV-3, and a new variant of GVA containing intriguing mutation. The results were published in two papers: one in the Journal of Phytopathology, the other in the Archives of Virology, in 2013 and 2014. Brief summaries of the results are presented below.

DISCOVERY OF A NEW HIGHLY DIVERGENT VARIANT OF GLRaV-3

Two sibling plants of Shiraz with different SD statuses, propagated from a single mother plant, were subjected to virus analysis using RT-nested PCR based on *Closteroviridae*- and *Flexiviridae* –specific degenerate primers. The focus was on viruses of the *Closteroviridae* family, with *Grapevine leafroll-associated virus 3* (GLRaV-3) as the type member. This economically most important virus is always present, along with various variants of *Grapevine virus A* (GVA) in SD-affected grapevines. Recent results obtained by Bester *et al*. (Virol J., 2013) revealed that, contrary to earlier knowledge (Jooste and Goszczynski, Vitis, 2005; Turturo *et al*., J. Gen. Virol., 2005; Jooste *at al*., Arch. Virol. ,2010), the genome of this virus is extensively heterogenic. Variants of the virus cluster into more than six (I-VI) phylogenetic groups. It is becoming clear that highly divergent variants of GLRaV-3, the genomes of which share only about 70% nt identity with previously known variants of this virus, comprise numerous distinct groups. Members of one of these groups (group VI) are common in vineyards in South Africa (Bester *et al*., Arch. Virol., 2011). Currently, the full genome sequences of members representing three highly divergent groups: GH11, NZ2 and Is139 are known (Maree *et al*., Front. Microbiol., 2013). The isolate GH11 was identified and sequenced in South Africa (Bester et al., Arch. Virol., 2011). The study reported here led to the discovery of another highly divergent variant of this virus in local vineyards. The variant was named GTG10 (Fig. 3). The data presented in Fig. 3, which include variants identified in local vineyards (boxed), indicate that the most precise data on genetic heterogeneity of this virus was generated in South Africa. This strongly contributes to reliable detection of this virus.

Fig.3.



DISCOVERY OF A NATURAL MUTANT OF GVA

The application of the RT nested-PCR of Dovas and Katis in comparative analysis of the viruses of the *Closteroviridae* and *Betaflexiviridae* families infecting two sibling grapevines cv. Shiraz contributed highly to the discovery of a new genetic variant of Grapevine virus A (GVA). The grapevines, both infected with GRSPaV, GLRaV-3, GVB and GVA were propagated from a single mother plant. One of them become infected with Shiraz disease (SD), which is highly destructive on noble grapevine cultivars Shiraz and Merlot in South Africa. The new GVA variant was not associated with SD, since it was present in both SD-affected and SD-free plants. However, unlike in an earlier study of grapevines affected by this disease, this GVA variant of group I strongly dominated co-infecting variant of group II associated with SD and a variant of group III. The variant, named I327-5, was mechanically transmitted from SD-affected grapevine to *Nicotiana benthamiana*, and its genome was fully sequenced. The sequence data revealed that the most distinctive genomic feature of the variant I327-5 is the deletion of 3 nucleotides in the region where ORF2 and ORF3 genes overlaps. These genes of GVA encode a 19.8 kDa protein, the function of which remains unknown, and a 31 kDa protein that is indispensable for the movement of the virus in plants. The alignment of amino acid sequences of these proteins encoded by variant I327-5 with the respective proteins encoded by other members of group I suggest that, as the result of the mutation neutral Threonine or Alanine, and strongly positively charged Glutamic acid, respectively, were removed from the proteins of GVA variant I327-5. Preliminary results suggest that the variant is exceptionally fit in SD-affected plants. If the suggested high fitness of I327-5 variant in SD-affected grapevines is confirmed, the cDNA clone of the variant could be used as a vector for transient expression of viral and plant genes in the molecular study of this destructive disease. Fig. 4 shows (A) Scheme of GVA genome and (B, C) amino acid sequence variability between proteins encoded by (B) ORF2 and (C) ORF3 of GVA variants of phylogenetic group I. Arrows indicate position of the mutation identified in variant I327-5.

Fig. 4.

B

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I327-5     MTSQECTELSEFLVHSSDSYSLSEGSLESLSYVQCVQLLSDLKSLGYQSIDSILYILGGGEAERFEIYRI     70
Is.151     ---rd--g-----g-g---cdsgs---g--------n---------------------------------     70
GTG11-1    ----n----n---g-g-s-n---g-------na--lh--r--rd---k-------------------f--     70

I327-5     FRRHGIGIGEALQLGVRKCLSNSPRSTQAILDDLLSRLGRSNAFLPSDLGAVKGELVVTFHSSRLSVDLY    140
Is.151     ----------------k-s-c-----ll-----------gs---------------a-----------    140
GTG11-1    ------------------s--y---g-ae--------i--gs----c-----r---i-----a-------    140

I327-5     VNNKKVVTRSIQAEGDINYVARRFAGYKGLILRA.RH                                  176
Is.151     ----------v-----ys------s---------t-y                                  177
GTG11-1    i-------kfa-----a--a-g--s-----v--ta--                                  177
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C

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I327-5     MSQEGSQGTKASSF.VQDIKVFSVKRNTRDLESLNKSLHRGDVYDTELIEKVFPRRTKKCVIHRELIVKD     69
Is.151     ------l-------ep------h---s-----t-----------n------------------kdv----     70
GTG11-1    -r----lv-----sepr-----n---------------------------------------k------     70

I327-5     GRVDCDLDIMDGGLDDIDEVEFPLYHVGCIVVALMPHGKNLQGKVAVEVLDTRLKDGSSRISRTLMDMSK    139
Is.151     ----------e-----n-e-------------------------s--------v--a------------    140
GTG11-1    ----------e-------------------------------------------aa-----------    140

I327-5     PLSACADFPGYFISTSDLLNGYTLHLSITTTDLQFVDGVHPFSVQLMSIGRFCREDMKTRYAVAEASKLL    209
Is.151     ---------------------------------------------------g--------it-t--m-    210
GTG11-1    ---------------------------------------------------g--------it----m-    210

I327-5     HQNILNAEGDGELIPRGVQVQKVPDTLVMPEVYETIKKLGLKTNGTLRQEGGDKGDSRRVGAGESPTN     277
Is.151     ------t-------------------------f-----f------------r----n----v------     278
GTG11-1    ----------------------------------------r--------------n--a------a-     278
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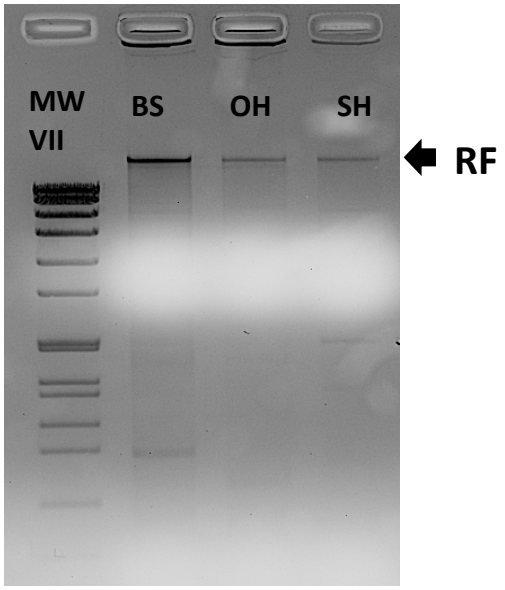
INVESTIGATION TO DETERMINE WHETHER RT-PCR AMPLIFICATION OF dsRNA USING A SINGLE PRIMER WITH RANDOM HEXANUCLEOTIDE SEQUENCE CAN BE USED FOR DETECTING GRAPEVINE VIRUSES

Almost all grapevine viruses have a single stranded RNA (ssRNA) genome. A double stranded RNA (dsRNA) form, which is an exact copy of the whole virus genome, called replicative form (RF), and its parts, are always present in plants infected with these viruses. The dsRNA is easy to isolate and relatively stable. It can be stored for years in a freezer at -20 ºC. This makes it a very good target in the detection and analysis of viruses. Although the dsRNA was also found in some virus-free plants, this is not common.

The dsRNA of any virus can be RT-PCR amplified using a single hexanucleotide primer. The primer used in the present study is composed of all possible combinations of four nucleotides in six nucleotide sequence at the 3' terminal part of the primer and the same 20 nt stabilising sequence at its 5' terminal part (5' GCCGGAGCTCTGCAGAATTC**NNNNNN** 3'). The variable hexanucleotide part of the primer will anneal to various places in any virus genome sequence, and act as primer in amplification of virus sequences by RT-PCR. The dsRNA has to be carefully isolated, since the hexanucleotide primer will also anneal to plant RNA contamination that is usually present in dsRNA samples.

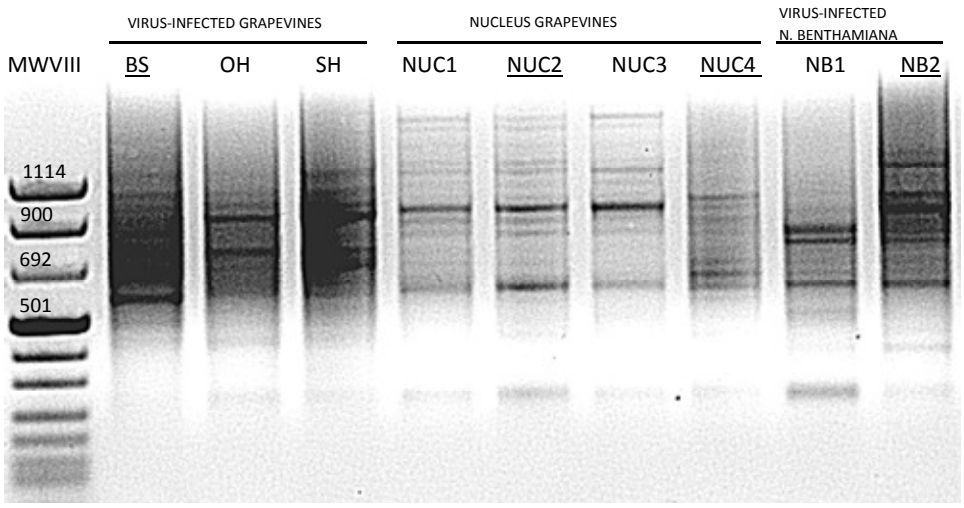
To overcome the possible amplification of plant RNA, the replicative form (RF) of viruses must be isolated. The sizes of RF for the various groups of viruses range from 6-19 kb. Results of the present study revealed that this approach can be applied for viruses infecting herbaceous plants, but is not practical for grapevines. The amount of dsRNA isolated from grapevines is much lower than for herbaceous hosts and, for some virus-infected grapevines, RF is hardly visible in electrophoretic gels. This makes the isolation of RF difficult. Figure 5 shows RF of closteroviruses isolated from grapevines cv. Black Spanish, Ohanez and Shiraz. The grapevines are infected with respectively GLRaV-1, -2, -3, -4; GLRaV-3, -6 and GLRaV-3, -4 (see Table 2). The grapevines are also infected with GRSPaV, GVA and GVB. The RF form of these viruses is about 3x smaller than closteroviruses, and is not visible in Fig. 5.

Fig. 5.



Thus, for grapevines the only option is to use total dsRNA in RT-PCR. In the trial, the dsRNA isolated from the following grapevine were used: cv. Black Spanish (BS), Ohanez (OH) and Shiraz (SH) from the PPRI collection, and four grapevines from the Vititec nucleus collection, NUC1, NUC2, NUC3, NUC4. In the study (see Fig. 1B) that used virus group-specific degenerate primers, these nucleus plants appeared to be free of closteroviruses, but infected with betaflexiviruses, most probably *Grapevine rupestris stem pitting associated virus* (GRSPaV). Results of the amplifications of dsRNA from these grapevines using an RT-PCR procedure based on a single hexanucleotide primer are shown in Fig. 6. A much stronger amplification of dsRNA was obtained for grapevines infected with multiple viruses: Black Spanish, Ohanez and Shiraz than for 4 nucleus plants. DsRNA samples from *Nicotiana benthamiana* that exhibited medium (NB1) and severe (NB2) symptoms of virus infection were also used in these amplifications. These two plants are infected with GVA alone (NB1) and with a combination of GVA and an unidentified virus (NB2).

Fig. 6.



As mentioned earlier, the technique will amplify plant RNA that has contaminated dsRNA samples. To investigate if the amplified sequences are viral in origin, the amplicons of Black Spanish, and nucleus plants NUC2 and NUC4 (Fig. 6, underlined) were cloned. Plasmids from randomly selected clones per plant were purified then, after overnight digestion with EcoRI (to release cloned sequences from plasmid), the samples were electrophoresed in agarose gels. The sequences with sizes higher than 500 bp (see report 2013) were sequenced. No virus sequences were found among cloned 12 sequences amplified from nucleus plants. In contrast, six viral sequences (GLRaV-1 and GLRaV-3) were found among 9 cloned sequences amplified from multiple virus-infected Black Spanish (BS). Contamination of the total dsRNA with plant RNA makes the method impractical for routine diagnosis of virus infections. It may, however, find application in the analysis of plants with virus-like symptoms, but which are negative for known viruses.

Complete the following table

Milestone	Target Date	Extension Date	Date Completed	Achievement
1. Adaptation of the technique using grapevines of various cultivars, which were known to be multi virus-infected. The work included cloning of the PCR amplicons, SSCP analysis of randomly selected clones, sequencing and computer-assisted analysis of obtained sequences	2012		July 2012	Obtained strong and virus-specific simultaneous amplifications of members of the Closteroviridae and Betaflexiviridae families
2. The application of the technique to comparative analysis of populations of viruses infecting grapevines affected by Shiraz disease	2013		July 2013	Discovery of a new highly divergent variant of GLRaV-3, and a new naturally mutated variant of GVA .
3. Investigation of RT-PCR technique based on single hexanucleotide primer, which is potentially able to amplify all viruses	2013		December 2013	The technique is not recommended for routine detection of viruses because is not virus-specific, and effectively amplifies plant RNA. It can however be used to detect viruses in grapevines with virus-like symptoms but which are negative for all known viruses.
4. Journal publication/s – final milestone	2014		March 2014	Two papers in Journal of Phytopathology and Archives of Virology (see below)

Conclusions

Results proved that the RT nested-PCR technique based on degenerate primers developed by Dovas and Katis (2003) has the capability to efficiently amplify all known and yet unknown viruses of the *Closteroviridae* and *Betaflexiviridae* families associated with LR and RW diseases. Analysis of sequences of amplicons revealed the presence of virus sequences only. Thus, amplifications are virus-specific. This implies that the RT nested-PCR amplicons, which are clearly visible in agarose gels, indicate the presence of viruses of the *Closteroviridae* or *Betaflexiviridae* families in tested grapevines.

There are two factors that may hamper the direct application of this technique to detecting of viruses in grapevines. Firstly, the technique efficiently amplifies GRSPaV, a member of the *Betaflexiviridae* family. The virus is very common in vineyards worldwide, with incidence ranging from 70 to100% of grapevines. Also, the virus is difficult to eliminate from plants. Thus, monitoring the virus status of grapevines using RT nested-PCR of Dovas and Katis using only positive amplification may show a confusingly high percentage of virus infection among tested plants which, in fact, would only be GRSPaV infection. Positive amplification of sequences of *Betaflexiviruses* from nucleus plants used in this study is, possibly, an illustration of this. The amplified sequences are most probably sequences of GRSPaV. The economic importance of the virus is not clear at present. Although the virus is reported as being associated with stem pitting symptoms in grapevines, firm proof of the pathogenic effect of this virus on grapevines is still required. The very common presence of this virus in grapevines suggests that it has low

impact on the productivity of vineyards. Secondly, the identification of viruses amplified in RT nested-PCR of Dovas and Katis requires the application of a number of techniques. This includes isolation of amplicons from agarose, cloning, SSCP analysis of clones and sequencing. The method is rather laborious, and can only be used to monitor the virus status of very important grapevines of the nucleus collection.

Technology development, products and patents

Indicate the commercial potential of this project, eg. Intellectual property rights or commercial product(s)

Suggestions for technology transfer

List any suggestions you may have for technology transfer

There is a need for the grapevine industry to establish "research and development" laboratory. This laboratory would evaluate and standardise virus-detection techniques developed in other laboratories, focusing on strict alignment of techniques with the industry needs.

Human resources development/training

Indicate the number and level (eg. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

Student level (BSc, MSc, PhD, Post doc)	Cost to Project
1.	
2.	
3.	
4.	
5.	

Publications (popular, press releases, semi-scientific, scientific)

Goszczynski DE (2013). Brief report of a new highly divergent variant of Grapevine leafroll-associated virus 3 (GLRaV-3). Journal of Phytopathology (doi: 10.111/jph.12139).

Goszczynski DE (2014). Complete genome sequence of a natural mutant of grapevine virus A (GVA). Archives of Virology (DOI 10.1007/s00805-014-2085-4).

Presentations/papers delivered

Total cost summary of the project

TOTAL COST IN REAL TERMS	COST	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
YEAR 1						69000		69000	138000
YEAR 2						83180		83180	166360
YEAR 3									
YEAR 4									
YEAR 5									
TOTAL						152180		152180	**304360**