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Primers designed for the detection of grapevine pathogens spreading with propagating material by quantitative real-time PCR

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Summary: Several grapevine pathogens are disseminated by propagating material as systemic, but latent infections. Their detection and identification have a basic importance in the production and handling of propagating stocks. Thus several sensitive and reliable diagnostic protocols mostly based on molecular techniques have been developed. Of these methods quantitative real-time PCR (q-PCR) has recently got an emerging importance. Here we collected primer data for the detection and identification of grapevine pathogens which are important in the production of propagating stocks by q-PCR. Additional novel techniques that use DNA amplification, hybridization and sequencing are also briefly reviewed.

Keywords: bacteria, fungi, phytoplasmas, polymerase chain reaction, primer sequences, viroids, viruses, *Vitis vinifera*

Introduction

The use of healthy planting stock is a basic prerequisite to prevent spreading of several grapevine diseases including viroids, viruses, phytoplasmas, bacteria and fungi which systemically and frequently infect their host plant in latent form. To obtain pathogen-free plants several protocols have been developed which include various diagnostic methods that include biological, -serological and molecular assays to detect and identify, and curative treatments to eliminate the various pathogens (Bisztray et al. 2012, Szegedi et al. 2012). Due to its high sensitivity, cost efficiency and easy application the polymerase chain reaction (PCR) has become the most widely used diagnostic protocol during the last 25 years in plant pathology (Louws et al. 1999). To increase the sensitivity, reliability and specificity of conventional PCR methods several novel technologies have been introduced into the plant quarantine studies during the recent years. In addition to increased sensitivity, most of these methods allow also the simultaneous detection of multiple pathogens.

Novel molecular diagnostic techniques

Loop-mediated isothermal amplification: In spite of the high convenience, sensitivity and cost-efficiency of conventional PCR several novel molecular techniques have been developed and introduced to increase the sensitivity, reliability and/or applicability of diagnostic and identification protocols. The loop-mediated isothermal amplification (LAMP) originally developed by Notomi and coworkers (2000) uses a set of inner and outer primers and *Bst* polymerase that amplifies DNA at 65 °C. The elongation is followed by a colourimetric reaction using, e. g., hydroxynaphthol blue (Goto et al. 2009). Thus the use of thermal cycler, gel electrophoretic separation and ethidium-bromide staining, and even DNA extraction is not necessary for scoring the results. Among grapevine pathogens this protocol has already been used for the detection of *Xylella fastidiosa* (Harper et al. 2010) and phytoplasmas (Kogovšek et al. 2015, Tomlinson et al. 2010). Further improvement includes addition of reverse transcriptase into the reaction which allows detection of RNA targets, RNA viruses and viroids. Among grapevine viruses an RT-LAMP based method was developed for GLRaV-3 and proved to be

as sensitive as nested PCR (Walsh and Pietersen, 2013). Besides its high specificity and sensitivity a great advantage of LAMP is that it can be easily used in mobile laboratories, for example in the field and state border quarantine stations.

Micro and macroarray techniques: The microarray and macroarray technologies involve the hybridization of PCR amplified and labelled samples to immobilized oligonucleotide probes specific for the various pathogens. Engel and coworkers (2010) printed 70-mer oligonucleotides of 570 probes specific for 44 grapevine viruses onto the surface of a microscope slide. These probes included both family-, and species-specific sequences. Thus each virus was covered by several specific sequences. Viral sequences were enriched by random primed PCR prior to hybridization. Using this step the detection becomes more sensitive than labelling total plant RNA. Since microarrays allow the detection of ten-thousands of specific sequences in a single hybridization step, this technique allows the detection of mixed infections or even the complete virus population present in the tested plant. Thompson and coworkers (2014) used the macroarray technology to detect and identify grapevine viruses. They used similarly 60-70-mer oligonucleotide virus-specific probes. Approximately 1600 probes specific for 38 viruses and plant internal controls were immobilized onto a nylon membrane and used for the subsequent hybridization experiment with PCR enriched and labelled plant cDNA. Although macroarrays allow the application of approximately only one tenth of probes than that of microarrays, it is still far sufficient to detect viruses infecting grapevines and the method does not require expensive equipments and the membrane blots can be reused up to 20-30 times. Results of the micro-, and macroarray detections were consistent with ELISA and/or reverse transcription PCR assays. The great advantages of array technologies over the conventional and real-time PCR that they cover much longer nucleotide sequences (several 70-mer oligonucleotides for a given virus) thus the reaction is highly specific for the given pathogen. In simple nucleic-acid amplification based detections the reactions are determined by short (usually 21-25 nucleotide long) primers thus a few mutations may cause false negative results. A further hybridization-based technique applied a „polyprobe” established from four tandemly cloned viroid sequences. This probe was then labelled with digoxigenin (DIG) and hybridized to grapevine RNA extract blotted onto nylon membrane. This method allowed the simultaneous detection of four viroids (Zhang et al. 2012). To detect fungal diseases Martos and coworkers (2011) dot-blotted the PCR products onto a nylon membrane followed by hybridization with a *Phaeomonniella*-specific probe. The low density-array uses microwell plates (with 384 sample wells) precoated with a set of virus specific primers and TaqMan probes thus allowing the simultaneous detection of several viruses from a large number of samples by quantitative real-time PCR (Osman et al. 2008).

Barcodes: A recent technique called barcoding combines PCR amplification and sequencing. A basic prerequisite of

a barcode is that it should contain highly conserved regions for a given taxonomic group but sequences bordered by these regions should be variable enough to discriminate species or strains. In the first step of barcoding a relatively short DNA fragment is amplified using primers designed for the conserved borders. Subsequently, this fragment is sequenced that allows the precise identification of the given organism. DNA barcodes for phytoplasmas based on *tuf* gene sequences (Makarova et al. 2012) and for fungi based on the ITS region (Schoch et al. 2012) have already been published, and barcode database for plant pathogens has also been established (Bonants et al. 2010, www.qbol.org). This database contains data for some grapevine pathogenic bacteria such as *Xylella fastidiosa* subsp. *fastidiosa* and *Xanthomonas viticola* as well.

Deep (Next generation) sequencing: Fast evolution in sequencing techniques established the possibility to get metagenomic information of the investigated plants. Next generation sequencing serves as a base of finding all of the expressed RNA (including the pathogens also) in the host and get a virome of the plant. Different platforms were used for the description of new grapevine viruses: Roche 454 for Grapevine Syrah-1 virus (Al Rwahnih et al. 2009), while Illumina for Grapevine virus F (Al Rwahnih et al. 2012, Al Rwahnih et al. 2013). This later platform was used also to create the viromes of a vineyard (Coetzee et al. 2010). RNA interference, the RNA based defense reaction of the plant, opens a new possibility in virus diagnosis using deep sequencing of small RNA libraries of infected plants (Donaire et al. 2009, Kreuze et al. 2009). During virus infection small interfering RNAs having sequence similarity to the infecting viruses are formed and sequencing them make it possible to identify viroids (Navarro et al. 2009) or viruses (Pantaleo et al. 2010) even if they are alien on the plant or never described (Giampetruzzi et al. 2012, Wu et al. 2012, Zhang et al. 2011). Deep sequencing offers a unique opportunity to reveal any viruses or viroids present in the sample, either expected or not. It needs a major investment at the beginning, running costs are relatively high, but barcoding the libraries for pooled sequencing, improvement of data analysing pipelines (Ho and Tzanetakis, 2014) and serious drop in the sequencing costs can make this method suitable for large scale sample testing in the near future.

Changes in the expression profile of grapevine host genes: Pathogen infections may cause specific changes in the expression profiles of infected grapevine plants (Albertazzi et al. 2009, Choi et al. 2010b, Espinosa et al. 2007, Fung et al. 2008, Hren et al. 2009, Santi et al. 2013, Zhao et al. 2011). Therefore analysis of host mRNAs may provide valuable information for pathogen diagnosis. Choi and coworkers (2010a) found four grapevine genes which were specifically up-regulated following *Xylella fastidiosa* infections, thus monitoring these genes indicates latent infections prior to symptom development. In symptomless *Eutypa lata*-infected grapevine plants 10 genes were found which were differentially up-, or down- regulated (Camps et al. 2014).

GC-MS analysis of volatile compounds in healthy and galled (*Agrobacterium vitis*-infected) grapevine cuttings provided further indirect evidence for the pathogen-induced changes in plant gene expression. Diseased plants accumulated styrene, a phenylalanine derivative that was not present in healthy samples (Blasioli et al. 2010). In phytoplasma infected grapevine leaves genes involved in plant defence mechanisms such as callose synthase, sugar transporters and cell wall invertase showed higher expression (Santi et al. 2013).

The quantitative real-time PCR: Soon after the introduction of conventional PCR a more developed technique, the quantitative real time-PCR (q-PCR) has also been introduced for the detection of plant pathogens (Boonham et al. 2014, Christensen et al. 2013, Hren et al. 2010, Schena et al. 2004). The q-PCR is more sensitive than the conventional PCR method and the amplification and detection is combined in one step, thus subsequent gel-electrophoretic separation of the amplified product is not necessary. The amplification is continuously detected by various fluorescent reporters

Table 1. Viroid specific primers

Viroid*	Primer	Primer sequence (5'-3')	At/Tm (°C)*	Fragment length (nt)	Probe	Reference
CEVd	CEVdF	GCGTCCAGCGGAGAAACA	60	68	TaqMan	Papayiannis 2014
	CEVdR	CAGCGACGATCGGATGTG	60			
	CEVdTAQ	FAM-TCGTCTCCTTCCTTCGGTCTGG-BHQ1	70			
HSVd	HSVdF	GCCTTCGAAACACCATCGA	60	71	TaqMan	Papayiannis 2014
	HSVdR	CACCGGTCGCGTCTCATC	60			
	HSVdTAQ	HEX-CGTCCCTTCTTCTTACCTTCTCTGGCTC-BHQ2	69			
HSVd	HSVd FP	CCGGGATCCTCTCTTGA	55	106	TaqMan	Sun et al. 2014
	HSVd RP	CCGGGGCTCCTTCTCAG				
	HSVd probe	FAM-CTGGGGAAATTCGAGTTGCCGCA-BHQ1				
HSVd	HSVd-H-long	CCGGGGCAACTTCTCAGAATCCAGCGAG	60	65	TaqMan	Luigi & Faggioli 2013
	HSVd-C-long	GAAGAAGGGACGATCGATGGTGTTCGAAG				
	HSVd-Probe	AGAGAGGGCCGCGGTGCTCT-BHQ1				
GYSVd1	GY FP	CTTGTGGTTCCTGTGGTTTCAC	55	69	TaqMan	Sun et al. 2014
	GY RP	CCTCTGCCCTATCTTCTTCTTT				
	GY probe	FAM-AGAGAGGGCCGCGGTGCTCT-BHQ1				

*CEVd = Citrus exocortis viroid, HSVd = Hop stunt viroid, GYSVd-1, = Grapevine yellow speckle viroid-1

Table 2. Virus specific primers

Virus*	Primer	Primer sequence (5'-3')**	At/Tm (°C)	Fragment length (nt)	Target gene	Probe	Reference
ArMV ^a	ArMV FP ArMV RP ArMV probe	GCACTGTAGCCCTTGGAGATAATCC CCCTCCAAATCCACATTAACCTTA CTCACATGATAGCTTGTCTAGGACTCC	55	100	RNA2- polyprotein	TaqMan	Sun et al. 2014
ArMV	ArMV i1 ArMV i2 ArMV p S3^b ArMV p ES^b	AATTATATGCTGAGTTTGAG AAAATTATACACCTTATGAGTA ACCAGTGCCTACAAGAGTGTGTCC ACCAGTGCCTATAAGAGTGTTC	60	203	RNA2- polyprotein CP	TaqMan	Bertolini et al. 2003 Bertolini et al. 2010
ArMV	ArMV III D ArMV III R ArMV III P	TAGCCCTTGTACTTATGGCA TATTTAAACAGTTGATTCCA TTGTTAGTGAATGGAACGGGGTCA	60	84	RNA2- polyprotein P2V	TaqMan	López-Fabuel et al. 2013
GfKv	GfKvPrep220F GfKvPrep261R GfKvPrep230P	ACGTGAAGACCAACGTGCAAT CGGTGATGCGCATGCA CCAATTTGGCCCTCTC	52	56	Replicase	TaqMan	Pacifico et al. 2011
GfKv ^c	Fleck 239 f Fleck 328 r Fleck 261 p1 Fleck 261 p2 Fleck 261 p3	CAACATCGAATGCCAATTTGG GCCAGGCTGTAGTCGGTGTGT CCTCTCACGTGCATGCGCATC CCTCTCACGTGCATGCGGATC CCTCTGACGTGCATGCGCATC	60	89	Replicase	TaqMan	Osman et al. 2008
GfKv	GfKv OB F GfKv OB R GfKv OB S	CGAGAACTCTCTTTACCTC CCGGCGTGGATGTAGAG ACCCTCGCCCTCATGCA	60	146	Replicase	TaqMan	Bertolini et al. 2010
GFLV	GFLV F GFLV R GFLV probe1^d GFLV probe2^d	AGCTGCGGCACTYTTTGC TCATCACTRGTCATACCACCTTCT TGCTCAARCATACCCTTG ATGCTTAARCATACCCTTG	60	128	RNA2- polyprotein 2AHP	Taqman	Čepin et al. 2010
GFLV	GFLVppoly 617F GFLVppoly 660R GFLVppoly 640P	CTCGTCCACTGCTGGAATTAGA ATCCTGCCTCCAGAGCTTTTT TTTTGAAACGTGGATTG	52	64	RNA1- polyprotein RdRP	TaqMan	Pacifico et al. 2011

Continuation of *Table 2*

Virus*	Primer	Primer sequence (5'-3')**	At/Tm (°C)	Fragment length (nt)	Target gene	Probe	Reference
GFLV	GFLV-769f GFLV-868r GFLV-799p	GGGACCACACTATGGATGGAATGA TTCCGGTGATATGGAGAGCGAAT AAGTATCCCGGGGTGTATGTGGAAGAGGA	60	99	RNA2- polyprotein CP	Taqman	<i>Osman & Rowhani</i> 2006
GFLV	GFLV-769f GFLV-868r GFLV-	GGGACCACACTATGGATGGAATGA TTCCGGTGATATGGAGAGCGAAT AGTGGAACGGGACCAC	60	99	RNA2- polyprotein CP	Taqman	<i>López-Fabuel et al.</i> 2013
GFLV	GFLV CP2-671f1 ^c GFLV CP2-671f2 ^c GFLV CP2-822 r GFLV CP2-761p	GTTGTGTGTAGGTATGGGAGGTACTATTA TGTGTTTTGGGTATGGGAGGTACTATTA TTCCACATACACCCGGGATA AGTGGAACGGGACCAC	60	149	RNA2- polyprotein CP	TaqMan	<i>Osman et al.</i> 2008.
GLRaV-1	GLRaV1Ppoly167F GLRaV1Ppoly206R GLRaV1Ppoly190P	CTGACCCTATCGCTGCTACTGA TCCCATCAACCCAGGTATCAA CTCGCCAGCTTGT	52	60	RNA dependent RNA polymerase	TaqMan	<i>Pacifico et al.</i> 2011
GLRaV-1	HSP70-149 F HSP70-293 R HSP70-225 P	ACCTGGTTGAACGAGATCGCTT GTAAACGGGTGTTCTTCAATCTCT ACGAGATATCTGTGGACGGA	60	144	HSP70-like protein	Taqman	<i>Osman et al.</i> 2007
GLRaV 2	198 F 290 R 233 P	CATTATATTCTTCATGCCTCTCAGGAT GATGACAACCTTGTCCGCTATAGC TTGCTACTGATCGACTGTGCAGCTCACA	60	92	HSP70-like protein	TaqMan	<i>Osman et al.,</i> 2007
GLRaV-2	P19qtF4 P24qtR	CTAACAAATTTCTTCTTGGATCGCAT AGAATGTCTTCAGCTTCATAAGGAG	61	154	p19 and p24 kDa protein	SYBR Green	<i>Beuve et al.</i> 2007
GLRaV2- RG	Redglobe-227 f Redglobe-319 r Redglobe-250 p	GCGACTCCAGCAACTTTAGTGA CCGTATCATTACAGACCAGTACCTATTT TACTTTGATCGCCTGAAGCCACACTATGC	60	92	HSP70-like protein	TaqMan	<i>Osman et al.</i> 2008
GRLaV-3	GLRaV3Ppoly36F GLRaV3Ppoly84R GLRaV3Ppoly54P	GGCGGAGGTGACGGAAA CCCTTTTGTCCAACCAATCT CCATTTGTCCAGCAACGCGACGT	52	67	RNA dependent RNA polymerase	TaqMan	<i>Pacifico et al.</i> 2011
GLRaV-3	GLRaV3-56f GLRaV3-285r GLRaV3-181p	AAGTGCTCTAGTTAAGGTCAGGAGTGA GTATTGGACTACCTTTCCGGAAAAT CAGGTAATAGCGGACTGAGACTGGTGGACA	60	229	HSP70-like protein	Taqman	<i>Osman & Rowhani</i> 2006
GLRaV-3	LR3_6995F LR3_7138R LR3_14586F LR3_14725R LR3_18345F LR_18488R	GGGRACGGARAAGTGTACC TCCAAYTGGGTCATRCACAAA	53	143	ORF1a	SYBR Green	<i>Bester et al.</i> 2014
		ATGAAYGARAARGTYATGGC CTAACGCYTYGTGYCTAG	50	139	Coat Protein		
		CCTCACGGTTTAATACTCTG ATTGTCGATAAGTTAGCCTC	54	143	3'UTR		
GLRaV-4	LR4 hsp-85 f LR4hsp-178 r LR4hsp-120 p	ATATACATACCAACCGTTGTGGGTATAA CCCTATAAACTAGCACATCTTCTCTAGT TGGAACATATACCATTGGGCTTGGTGTCT	60	93	HSP70- like protein	TaqMan	<i>Osman et al.</i> 2007
GLRaV-5	GLRaV-5 26 f GLRaV-5 188 r GLRaV-5 131p	AACACTCTGCTTTTCTGCTGGC CTTTTTATGTCCCGATAAACGAGTACA CAATAGGTTTGGGGCTTGTCTAGAAAAGG	60	161	HSP70	TaqMan	<i>Osman et al.</i> 2007
GLRaV-9	LR9-114 f LR9-196 r LR9-136 p	CGGCATAAGAAAAGATGGCAC TCTTTATGTACGGTAGACCAACAC TATACCATAGGATTGGGAGCTCTGCTGGAGA	60	82	HSP70	TaqMan	<i>Osman et al.</i> 2007
GVA	GVAPrep121F GVAPrep169R GVAPrep143P	AATGTCATGGACTGGGTGCGAA GATATGTGCATGAGATTCAC ATGACAGCGCGAACCCCTCAGTG	52	73	Replicase (RdRp)	TaqMan	<i>Pacifico et al.</i> 2011
GVA ^f	GVA-77 f1 GVA-77 f2 GVA-192 r1 GVA-192 r2 GVA-104 p	CGACCGAAATATGTACTTGAATACTC CGACCGAACTATGTACTTGAATACTC TTTGCTAGCTTTAGGACCTACTATATCTACCT CTTGCTAGCCTTAGGTCCTACTATATCTACCT CTTCGGGTACATCGCCTTGGTCCG	60	111	Coat protein	TaqMan	<i>Osman & Rowhani</i> 2008
GVA [*]	GVAmu-77f1 GVAmu-77f2 GVAmu-192r1 ^h GVAmu-192r2 ^h GVAmu-104p	CGACCGAAATATGTACTTGAATACTC CGACCGAACTATGTACTTGAATACTC AGGTAGATATAGTAGGTCCTAAAGCTAGCAA GGTAGATATAGTAGTAGGACCTAAGGCTAGCAA TCGGGTACATCGCCTTG	58	110	Coat protein	TaqMan	<i>Osman et al.</i> 2013
GVB ^f	GVB-92 f1 GVB-92 f2 GVB-95 f3 GVB-202 r1 GVB-202 r2 GVB-119 p1 GVB-119 p2	CTAGGAGTGCGGCTAAACGAA GGAGTGCGGCAAAACGA CAAGGAGTGCGGCTAAACGAA CCTAACCTCGCTGTGATATGGT CCTTCACCTCATCYTGGGATCGTGT CTCGTTATGGTTCGCTGTACTGTTGTGGTAG ACCGTTACGGCCGTGTTACTGTTGTGGTAG	60	110	RNA binding protein	TaqMan	<i>Osman & Rowhani</i> 2008

Continuation of **Table 2**

Virus*	Primer	Primer sequence (5'-3')**	At/Tm (°C)	Fragment length (nt)	Target gene	Probe	Reference
GVB [§]	GVBmu-91f1 ^j GVBmu-91f2 ^j GVBmu-91f3 ^j GVBmu-202r1 ^h GVBmu-202r2 ^h GVBmu-119p1 GVBmu-119p2	CTAGGAGTGCGGCTAAACGAA GGAGTGCGGCCAAACG CAAGGAGTGCGGCTAAACG CCATATCACAGGACGAGGTTAAGG ACGATCCCAYGATGAGGTGAA TGGTCGCTGTTACTGTT ACCGTTACGGCCGTTG	58	108	RNA binding protein	TaqMan	Osman et al. 2013
GVD	GVD-554 f GVD-631 r GVD-580 p	AGGTGTATTCAACGCCAGTCG CCTGCGAGAAATGATGGGTCA TTGGAGAGCAGGCAGTTGAGATATAAATGGA	60	77	Coat Protein RNA binding protein	TaqMan	Osman & Rowhani 2008
GVD [§]	GVDmu-554f GVDmu-661r ^h GVDmu-581p	AGGTGTATTCAACGCCAGTC GTAGTTATGCTAAGAAGCGTAGGGC TGGAGAGCAGGCAGTT	58	107	Coat Protein, RNA binding protein	TaqMan	Osman et al. 2013
GRSPaV ^f	RSPaV-52 f1 RSPaV-52 f2 RSPaV-52 f3 RSPaV-130 r1 RSPaV-130 r2 RSPaV-75 p1 RSPaV-75 p2 RSPaV-75 p3	AGACGGGAATACCACCAGCTAA AGACGGGAATTCCACCCGCTAA AGACGGGATACCACCAGCTAA AGGAAGAAGTCAAAGGCTGCAA AAGAAAAAATCAAAGGTGCAA TGGGCCAAGAAAGGATTTAATGAGAATGAA AAG TGGGCCAAGAAAGGGTTAATGAGAATGAA AAA TGGGCCAAGAAGGGATTTAATGAAAATGAG AAA	60	78	Coat protein	TaqMan	Osman & Rowhani 2008
TBRV	TBRV-70F ⁱ TBRV-70R ⁱ TBRV-70P	GCTCGTAACAGTTGCGGAGATAT TGTCACACTGTCATGGGA TGCATAGGCTCACTCCTTGGGA	62	72	RNA2- Polyprotein	TaqMan	Harper et al. 2011
ToRSV	ToRSV-UTRf ToRSV-UTRr ToRSV-UTRp	GAATGGTTCCCAGCCACT AGTCTCACTTAACATACCAC AGGATCGCTACTCCTCCGTCACAC	60	182	RNA2-3' UTR		Lebas & Ward 2012
ToRSV	ToRSV-1590 f ToRSV-1661 r ToRSV-1610 p	GCCACCCGAGAACGTTAGC GCCTGCTGAGTCTGCTGTAGAG CCACGGGCCCGGTAGTCAATATG	60	71	RNA2- Polyprotein-CP	TaqMan	Osman et al. 2008

*ArMV: Arabis mosaic virus, GFLV: Grapevine fanleaf virus, GFkV: Grapevine fleck virus, GLRaV-1, -2, -3, -4, -5, and -9: Grapevine leafroll-associated virus-1, -2, -3, -4, -5, and -9, respectively, GRSPaV: Grapevine rupestris stem pitting-associated virus, GVA: Grapevine virus A, GVB: Grapevine virus B, GVD: Grapevine virus D, TBRV: Tomato black ring virus, ToRSV: Tomato ringspot virus

** For TaqMan oligonucleotide probes (show in bold) we only refer for the sequence and not show either the fluorophore or the quencher.

[§]ARMV in this case was tested in lily leaf not in grapevine

^htwo different probes mixed together were used for successful detection of highly variable isolates

ⁱIn order to increase sensitivity different probes were used to detect all or at least the majority of diverse isolates

^jTwo different probes were used separately

^hTwo different forward primers mixed together were used for successful detection of variable isolates

^fThe mixture of the multiple primers were used to increase the efficiency of the detection

[§]Oligos were used in different combinations

^hBe aware that these published reverse oligos are in sense orientation, for PCR reaction reverse complement of them must be used

^jBe aware that these published forward and reverse oligos are mixed up

^jThe forward oligos are the same as in the previous paper

bound to the primers (Molecular beacon and Scorpion probes) or to internal complement oligonucleotide sequences (TaqMan[®] probes) or bound specifically only to the amplified double stranded DNA (SYBR[®] Green). Until annealing or insertion of primer or probe sequences to the target DNA the light emission is blocked by specific quenchers. The quenchers are removed during amplification from the reporters allowing the fluorescent light emission. Due to its high sensitivity and specificity the application of q-PCR has been widely used in plant quarantine laboratories despite of its relatively high costs.

At present the various versions (TaqMan[®], SYBR Green[®]) of q-PCR protocols combined with traditional PCR are most extensively applied in pathogen detection and identification. In a previous survey we have collected a primer „databank” for the detection of the most important grapevine pathogens by conventional PCR methods (*Manduláné Farkas et al. 2014*). As a subsequent part of this work here we summarize primer data available for q-PCR detection of grapevine pathogens spreading with propagating material (Tables 1–5).

Table 3. Phytoplasma specific primers

Phytoplasma	Primer	Primer sequence (5'-3')	At/Tm (°C)	Fragment length (nt)	Target gene	Probe	Reference
Universal	Forward Reverse Probe	CGTACGCAAGTATGAACTTAAAGGA TCTTCGAATTAACAACATGATCCA FAM-TGACGGGACTCCGCACAAGCG-TAMRA	60	n. g.*	16SrRNA	TaqMan	Christensen et al. 2004
Universal	UniRNafw UniRNarev Probe	AAATATAGTGGAGTTATCAGGGATACAG AACCTAACATCTCAGACACGAACT FAM-ACGACAACCATGCACCA-NFQ**	60	73	16SrRNA	TaqMan	Hren et al. 2007
Bois noir Stolbur 16SrXII-A Tuf-type a	FStol4-tA RStol4-tA Probe	GATCCACCCTTCGCTTTATTT CTTGAATAACTGAAAGCGACA FAM-GTCAAACACCACCTTTTATCATTCCT-BHQ-1	60	222	Stol4 fragment	TaqMan	Daire et al. 1997, Fahrentrapp et al. 2013
Bois noir Stolbur 16SrXII-A Tuf-type b	FStol4-tB RStol4-tB Probe	GATCCACCCTTCGCTTTATTT CTTGAATAACTGAAAGCGACA HEX-ATCAAAAACAACAACCTTTTATCATTC- BHQ-1	60	222	Stol4	TaqMan	Fahrentrapp et al. 2013
Bois noir Stolbur 16SrXII-A	mapBN-F mapBN-R mapBN-VIC	ATTTGATGAAACACGCTGGATTAA TCCCTGGAACAATAAAAAGTYGCA VIC-AAACCACAAAATGC	60	72	map	TaqMan	Pelletier et al. 2009
Bois noir Stolbur 16SrXII-A	190F 660R 210F 280R BN-P	GAGATAAGAAGGCATCTTCTTA AACAGTTTTTATAGCATCACAA CTTCTATTTTTAAAAGACCTAGCAATAGG GTCTTGGTAGGCCATTACCC FAM-TTAGGGAAGAGCTTGCGTCA-BHQ-1	53 58	470 70	16S rRNA	TaqMan	Margaria et al. 2009***
Bois noir Stolbur 16SrXII-A	F R P	GGTTAAGTCCCGCAACGAG CCCACCTTCCTCCAATTTATCA FAM-AACCCTTGTTAATTGCCATCATTAAG- TAMRA	60	98	16S rRNA	TaqMan	Angelini et al. 2007
Bois noir Stolbur 16SrXII-A	F R P	AAGCAGGTTAGCGATGGTTGT TGGTACCGTTGCTTCATCATTT FAM-TTAATACCACCTTCAGGAAA-NFQ**	60	71	Stol11 genomic fragments	TaqMan	Hren et al. 2007
BN 16SrXII-A-VK_I	qBN-AD-F qBN-AD-R qBN-VKI	CCTTCTTAATGCCAGTCGAA TGTCCTCTTCAACTCTACCAGTAA FAM-TCCTCTACCGGTGATAG	57	80	tuf	TaqMan	Berger et al. 2009
BN 16SrXII-A-VK-II	qBN-AD-F qBN-AD-R qBN-VKII	CCTTCTTAATGCCAGTCGAA TGTCCTCTTCAACTCTACCAGTAA VIC-TGTTCTCTACCAGTGAT	57	80	tuf	TaqMan	Berger et al. 2009
Flavescence dorée 16SrV-C, D and E	mapFD-F mapFD-R mapFD-FAM	TCAAGGCTTCGGBGGTTATA TTGTTTTAGAAAGTAATCCGTGAACTAC FAM-TTGTATTTCAAGTGAATGAAG	60	71	map	TaqMan	Pelletier et al. 2009
FD	395F 480R FD-P	GCCGCGTGAACGATGAA GAATAACGTCAAGATAGTTTTTCCACT FAM-TTTCGGTATGTAAAGTTCT-TAMRA	58	85	16SrRNA	TaqMan	Margaria et al. 2009
FD	F R P	AAGTCGAACGAGACCCTTC TAGCAACCGTTTCCGATTGT FAM-AAAAGGTCTTAGTGGCGAACGGGT-TAMRA	60	103	16SrRNA	TaqMan	Angelini et al. 2007
FD	F R P	TTATGCCTTATGTTACTGCTTCTATTGTTA TCTCCTTGTTCTTGCCATCTTT FAM-ACCTTTTGACTCAATTGA-NFQ	60	85	secY	TaqMan	Hren et al. 2007
FD 16SrV-D, C	F1024 F1112 probe	GTGAGATGTTAGGTAAAGTCCTAAAACGA GGACTTTAGCGAGACTGCCAA FAM-AACCCCTGTCTGCTAGTTGCCAGC-TAMRA	60	89	16SrRNA	TaqMan	Bianco et al. 2004
Aster Yellows 16SrI	Forward Reverse Probe	TTGGGTTAAGTCCCGCAAC CCCACCTTCCTCCAATTTATCA FAM-CCAGCACGTAATGGTGGGGACTT -TAMRA	60	102	16SrRNA	TaqMan	Angelini et al. 2007

*n. g.: not given, **: non fluorescent MGB quencher, ***: In BN detection, due to the low sensitivity of the 210F/280R primers, prior to qPCR an RT-PCR has to be performed using 190F/660R primers.

Table 4. Bacterium specific primers

Species	Primer	Primer sequence (5'-3')	At/Tm (°C)	Fragment length (nt)*	Target gene*	Probe	Reference
<i>Xylophilus ampelinus</i>	Xamp 14F Xamp 104R Xamp14F /104MGB	CCCGATGATAAAATACCGAAAACCTC TGTCTTCTGGTTGTTTTGGTTTTAAT FAM-AGCGCCTGACGCAT-MGB	60	91	Xamp 1.27A	TaqMan	Dreo et al. 2007
<i>Xylella fastidiosa</i>	XF-F XF-R XF-P	CACGGCTGGTAACGGAAGA GGGTTGCGTGGTGAATCAAG TCGCATCCCGTGGCTCAGTCC	62	70	rimM	TaqMan	Harper et al. 2010
<i>Xylella fastidiosa</i>	XfF1 XfR1 XfP1	AAAAATCGCCAACATAAAACCCA CCAGGCGTCCTCACAAGTTAC FAM- ACCTATGCCAACATCAACCCTGAATGCA- TAMRA	62	n. g.	ITS	TaqMan	Schaad et al. 2002
<i>Xylella fastidiosa</i>	XfF2 XfR2 XfP2	CTCGCCACCCATGGTATTACTAC CTGGCGGCAGGCCTAAC 6FAM-ATGTGCTGCCGTCCGACTTGCATG- TAMRA	62	n. g.	16S	TaqMan	Schaad et al. 2002
<i>Xylella fastidiosa</i>	HL5 f HL6 r probe	AAGGCAATAAACGCGCACTA GGTTTGTCTGACTGGCAACA FAM-TGGCAGGCAGCAACGATACGGCT- BHQ1	60	221	n. g.	TaqMan	Francis et al. 2006
<i>Agrobacterium vitis</i>	VIRD59F26 VIRD59R122	ATTGGAATATCTGTCCCG GGCGAGATCGCGGATATT	52	96	virD2	SYBR Green	Bini et al. 2008
<i>Agrobacterium vitis</i>	VIRD62F23 VIRD62R135	AACCATTCAGCAGGTAT TGGAATATGATCAGGCCG	52	102	virD2	SYBR Green	Bini et al. 2008
<i>Agrobacterium vitis</i>	virD2.For1 virD2.Rev1	TTGGAATATCTGTCCCGAAG CTTGATACCAGCAGGGAAGCTTA	50	n.g.	virD2	SYBR Green	Johnson et al. 2013

*n. g. = not given

Table 5. Fungus specific primers

Fungus	Primer	Primer sequence (5'-3')	At/Tm (°C)	Fragment length (nt)	Target gene	Probe	Reference
<i>Phaeoconiella chlamydospora</i>	pchITS417F pchITS495R pchITS439P	TGGCGGCGTCAAGAATGA CAATAGGGCCAGGCCAAG AGGTGCAGCGAGCAATCAAGCATACA	60	78	ITS2 region	TaqMan	Martin et al. 2012
<i>Phaeoconiella chlamydospora</i>	Pmo1f Pmo2r	GTTACATGTGACGTCTGAACG CAGTGTATGCTTGATTGCTCG	56	320	ITS region	SYBR Green	Overton et al. 2004
<i>Phaeoacremonium aleophilum</i> (Pa), <i>P. parasiticum</i> (Pp), <i>P. mortoniae</i> (Pm) and <i>P. viticola</i> (Pv)	F1btfw F2btfw R1bt Probe-Pa Probe-Pp Probe-Pm Probe-Pv	CCCTGARTTACCCACCATC VAGCTTCGACRWCTCGACG GCTACTTACRCAYTGRCGGTCTG FAM-CAGAATCTACCCAGATCATCGA CCAGC- 6-TAMRA TET-CGACTCTGACCCAAAAGCATCGAC-6- TAMRA VIC-CGTGAGCGTCACCTCTAAGTCATTGA CC-6-TAMRA VIC-CAAAATCAATTCAAATCATTGAACAGCT TGCTA-6-TAMRA	55	approx. 130-170	B-tubulin gene	TaqMan	Aroca et al. 2008
<i>Phaeoacremonium aleophilum</i>	palF11 palR11 pall1probe	CCTCGACGAGCCAGAATC GTAAAAACCCACCGGTTAGC CCCAGATCATCGACC	60	63	B-tubulin gene	TaqMan	Martin et al. 2012
<i>Phaeoacremonium</i> spp.	Pac1f Pac2r	ACCCTTTGTGAACATACCTG TACTGCGCTCGGAGTCTCTG	56	410-428	ITS region	SYBR Green	Overton et al. 2004
<i>Rosellinia necatrix</i>	R10fw R7rev	R10 CCCCTG TTG CTTAGT GTTGG R7 AACCATAGGCGAGATGAGAAAT	60	112	ITS2	Scorpion	Schena et al. 2002
<i>Rosellinia necatrix</i>	R15fw R18rev	CCATAGGCGAGATGAGAAATC CAGCCCCCTCGAAGTCAGT	58	71	ITS2	Scorpion	Schena & Ippolito 2003
<i>Rosellinia necatrix</i>	R2 fw R5rev TR2-5 probe	CAAAACCCATGTGAACATACCA CAATGCTAAACAGAGTTTCGTG 6-FAM-GTTAGGGCCTACCCGGTGGG-TAMRA	60	133	ITS1	TaqMan	Shishido et al. 2012*
<i>Rosellinia necatrix</i>	R10 fw R7rev TR10-7 probe	CCCCTG TTG CTTAGT GTTGG AACCATAGGCGAGATGAGAAAT 6-FAM-AGTCAGTGGCGGAGTCCGGTC-TAMRA	60	112	ITS2	TaqMan	Shishido et al. 2012*

*forward and reverse primers are from Shena et al. 2002

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