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Multiplex PCR for Identification and Detection of Cassava Mosaic Begomoviruses in Togo

Senya Sakina Allado[1*], Djodji Kossikouma Adjata[1], Justin Simon Pita[2], Assion Sétu Mivedor[1], Kodjovi Atassé Dansou-Kodjo[1], Koffi Tozo[3]

[1]Laboratoire de Virologie et de Biotechnologies Végétales, Ecole Supérieure d'Agronomie (ESA), Université de Lomé, Lomé, Togo
[2]Laboratory of Plant Virology, Université Félix Houphouët-Boigny, Pôle Scientifique et d'Innovation, Bingerville, Côte d'Ivoire
[3]Laboratoire de Physiologie et Biotechnologies Végétales, Faculté des Sciences, Université de Lomé, Lomé, Togo
Email: *senyasakina@gmail.com

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Abstract

Cassava mosaic disease (CMD) caused by Cassava Mosaic Begomoviruses (CMBs) is one of the most devastating crop diseases and a major constraint for cassava production. In order to ensure surveillance for epidemic prevention, low-cost diagnostic tools are appropriate for large-scale testing of cassava viruses. Multiplex PCR diagnosis is one approach that can reduce diagnostic costs and delays. A multiplex PCR approach was developed for simultaneous detection of African cassava mosaic virus (ACMV), East African Cassava Mosaic Virus and East African cassava mosaic Cameroon virus (EACMV/CM) in Togo CMD-infected cassava leaves. Three primers pairs were used to target their respective viruses in a single tube PCR. Multiplex PCR detected ACMV, EACMV and EACMV/CM in plant DNA extracts prepared from cassava leaves infected with CMB. The primers amplified 783 bp specific to ACMV, 650 bp specific to EACMV and 560 bp specific to EACMCV/CM in both uniplex and multiplex formats. Multiplex PCR is an excellent tool for the effective control of cassava diseases.

Keywords

Cassava, *Begomovirus*, CMD, CMB, EACMV/CM, PCR Multiplex

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a vegetatively propagated staple crop of great economic importance. Due to its inherent tolerance to drought and inadequate soil nutrients, cassava produces some storage roots where other food crops would fail; hence, it is considered a food security crop [1] [2]. It provides food security in several African countries [3], as it is easy to grow and its yields help

to survive during famine [4]. The food security and livelihood benefits of cassava in Africa are, however, constrained by cassava mosaic disease (CMD). It is caused by Cassava Mosaic Begomoviruses (CMBs) transmitted by the whitefly vector Bemisia tabaci [5] and are also disseminated through infected stem cuttings used for new plantings, since cassava is a crop propagated vegetatively. Several cassava infecting Begomoviruses have been reported occurring in Sub-Saharan Africa [6] but only three are highlighted in this study. They are: African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), East African cassava mosaic Cameroon virus (EACMV/CM). ACMV and EACMV has been associated with a pandemic in East Africa [7] [8]. CMBs can occur in an infected plant either alone or as mixed infections of different combinations [9] [10] [11].

CMBs were detected by uniplex PCR, the usual method, using species-specific primers. Uniplex PCR are disadvantage of cost for a large-scale diagnosis of plants viruses. In recent years multiplex-PCR assay was developed by some authors [12] [13] using primers targeting replicase and coat protein regions of DNA component for specific and simultaneous detection of CMBs. To overcome the inherent disadvantage of cost, as well as to improve diagnostic capacity, multiplex PCR, a PCR variant in which more than one target sequence is amplified using more than one pair of primers, is an interesting alternative [14] to facilitate high throughput diagnosis of CMBs in epidemiological studies. The aim of this study is to develop a multiplex PCR approach for the detection of ACMV, EACMV and EACMV/CM in Togo CMD-infected cassava leaves.

2. Material and Methods

2.1. Collection of Virus-Infected Cassava Leaves

In August-September 2020, cassava leaves with characteristic CMD symptoms and CMD-free were collected from farmers' fields located at intervals of between 10 km in five (05) agroecological zones of Togo. The latitude and longitude of each farm were recorded using Global Positioning System equipment (Garmin GPSMAP 64 s). Symptomatic and asymptomatic samples were collected and kept in the envelopes and dried at 25˚C.

2.2. Extraction of Total DNA

Total DNAs were extracted from 100 mg of leaf tissue of infected plants. Total DNA was extracted from leaf samples using InnuPREP Plant DNA Kit (Endress + hauser Compagny, Germany) according to the manufacturer's instructions. The extracted DNA was stored at −20˚C for analysis. The concentration and purity of extracted DNA samples were determined using a Nano Drop spectrophotometer (Thermo Scientific, Nano drop-2000C, Germany).

2.3. Primer Design Used in the PCR

The primers JSP001/JSP002 [8] identifying ACMV amplify a 783 bp fragment;

for EACMV primers CMBRep F/EACMVRep R [12] amplify a 650 bp fragment; and for EACMV/CM primers VNF031/VNF032 [15] amplify a 560 bp fragment (Table 1).

2.4. Single PCR Performance

The single (uniplex) assays were performed to detect each virus using the primer pair in the Table 1 designed to detect ACMV and EACMV. The samples were subjected to another round of PCR using specific primers for the detection of EACMV/CM (VNF031/VNF032). The reaction mixtures (25 µl) contained 12.5 µL Master Mix (New England Biolab, NEB), 1.25 µL each of forward and reverse primers (10 µM), 8 µL nuclease free PCR water (Inqaba Biotech West Africa Ltd) and 2 µL DNA. The PCR conditions used are those described by the various authors (Table 1).

2.5. Multiplex PCR Performance

The multiplex PCR for simultaneous detection ACMV, EACMV and EACMV/CM reaction conditions were optimized by using different primers concentrations, annealing temperatures, extension times to achieve optimum conditions (Table 2). The multiplex PCR reaction was set up in one tube as a 25 µl mixture containing 1 µl each of forward and reverse primers (10 µM), 5 µL nuclease free PCR water (Inqaba Biotech West Africa Ltd.), 12 µL Master Mix (New England Biolab, NEB), and 2 µL DNA.

Table 1. Oligonucleotide primer sequences for the single PCR.

Primer Name	Virus	Primer Sequence (5'-3')	Tm (˚C)	Amplicon (bp)	References
JSP001	ACMV	ATGTCGAAGCGACCAGGAGAT	55	783	8
JSP002		TGTTTATTAATTGCCAATACT			
CMBRep F	EACMV	CRTCAATGACGTTGTACCA	56	650	12
EACMVRep R		GGTTTGCAGAGAACTACATC			
VNF031	EACMV/CM	GGATACAGATAGGGTTCCCAC	52	560	15
VNF032		GACGAGGACAAGAATTCCAAT			

Table 2. Oligonucleotide primer sequences for the multiplex PCR.

Primer Name	Virus	Primer Sequence (5'-3')	Tm (˚C)	Amplicon (bp)	References
JSP001	ACMV	ATGTCGAAGCGACCAGGAGAT	52	783	8
JSP002		TGTTTATTAATTGCCAATACT			
CMBRep F	EACMV	CRTCAATGACGTTGTACCA		650	12
EACMVRep R		GGTTTGCAGAGAACTACATC			
VNF031	EACMV/CM	GGATACAGATAGGGTTCCCAC		560	15
VNF032		GACGAGGACAAGAATTCCAAT			

The PCR protocol started at 94˚C for 4 min followed by 35 cycles of amplification (94˚C for 1 min, 52˚C for 1 min, 72˚C for 1 min) and a final extension at 72˚C for 10 min. The DNA amplification was carried out in the Applied Bio systems Thermal Cycler. Amplicons were separated by electrophoresis on 1% agarose gels (which was stained with ethidium bromide) for 100 V for 35 min for uniplex PCR and 45 min for multiplex. Bands were viewed under UV light using and the images saved using a gel documentation system (MultiDoc-It Digital Imaging System).

3. Results

3.1. Single PCR for ACMV, EACMV, EACMV/CM

Uniplex amplification bands with expected sizes of 560 bp, 650 bp and 783 bp were obtained from the cassava samples with VNF031/VNF032, CMBRepF/EACMVRepR and JSP001/JSP002 respectively (Figure 1). ACMV were detected on 14/38, EACMV on 31/38 and EACMV/CM on 37/38 (Table 3). The volume of consumables used was 1710 µL (except DNA and nuclease free water) and the number of reactions to detect each of the viruses in uniplex is 114 reactions.

3.2. Multiplex PCR for ACMV, EACMV, EACMV/CM

Three amplification bands with expected sizes were obtained from the mixture of PCR products (Figure 2). ACMV were detected on 12/38, EACMV on 31/38, EACMV/CM on 37/38 (Table 3). The volume of consumables used was 684 µL (except DNA and nuclease free water) or 40% of the uniplex PCR and the number of reactions to detect each of the viruses in multiplex is 38 reactions.

The primers detected the three viruses in 12 of the 38 samples and EACMV, EACMV/CM were detected in 31 samples and none in one sample.

3.3. Comparison between Single and Multiplex PCR

Single and multiplex PCR detected ACMV, EACMV and EACMV/CM in plant DNA extracts prepared from cassava leaves infected with either two or three viruses. The primers JSP001/JSP002 amplified 783 bp specific to ACMV, primers CMBRepF/EACMVRepR amplified 650 bp specific to EACMV and primers VNF031/VNF032 amplified 560 bp specific to EACMCV/CM in both uniplex

Table 3. Results obtained by uniplex and multiplex PCR.

Virus	Number of samples with indicated result (positives number/total Number)	
	Uniplex PCR	Multiplex PCR
ACMV	14/38	12/38
EACMV	31/38	31/38
EACMV/CM	37/38	37/38

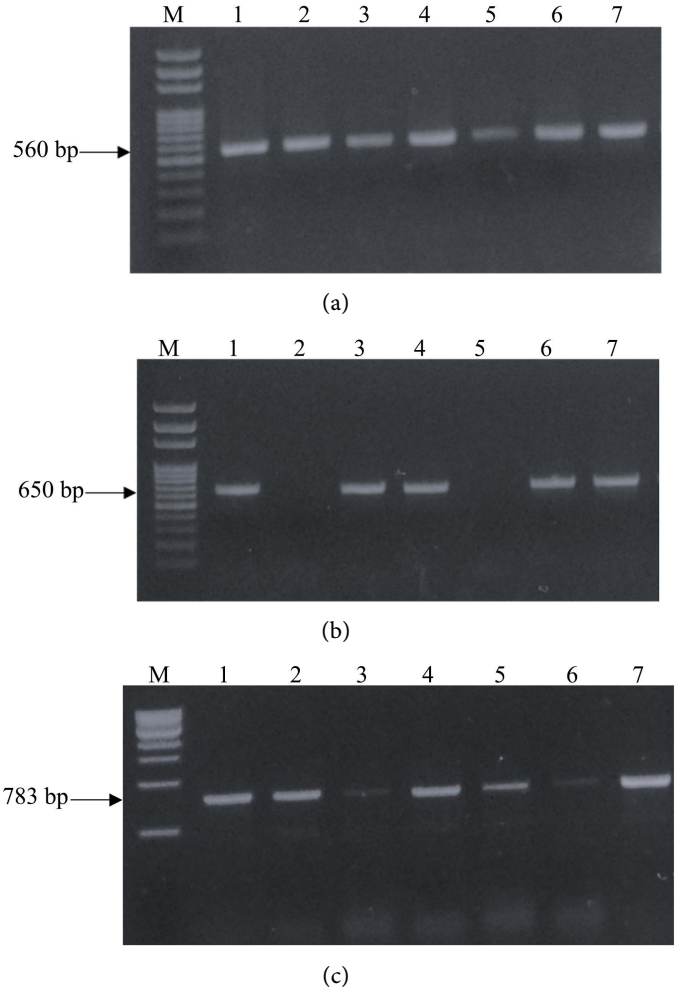

Figure 1. (a) Detection of EACMV/CM virus by PCR using specific primer VNF031/VNF032. Virus-specific amplification products correspond to 560 bp for EACMV/CM virus (Lane 1, 2, 3, 4, 5, 6 and 7). Lane M: 100 bp or 1 kb DNA ladder; (b) Detection of EACMV virus by PCR using specific primer CMBRepF/EACMVRepR. Virus-specific amplification products correspond to 650 bp for EACMV virus (Lane 1, 3, 4, 6, 7). Lane M: 100 bp or 1 kb DNA ladder; (c) Detection of ACMV virus by PCR using specific primer JSP001/JSP002. Virus-specific amplification products correspond to 783 bp for ACMV virus (Lane 1, 2, 3, 4, 5, 6, 7, 8). Lane M: 100 bp or 1 kb DNA ladder.

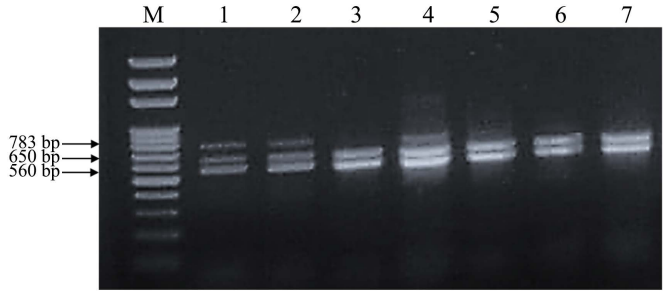

Figure 2. Simultaneous detection of all possible combinations of three viruses by multiplex PCR. Lane M: 100 bp or 1 kb DNA ladder; Lane 1, 2 and 4: for EACMV, ACMV and EACMV/CM respectivily; Lane 3, 5, 6 and 7: 650 and 560 for ACMV and EACMV/CM respectively.

and multiplex formats. Distinct sizes enabled the differentiation of virus-specific DNA bands by agarose from all samples Multiplex and uniplex obtained consistent results, except for ACMV where two less than those positive for primers JSP001/JSP002 using multiplex. Multiplex and uniplex results indicated that the former was relatively reliable for detecting mixed infection in the samples.

4. Discussion

In this study, a multiplex PCR was performed in order to simultaneously detect and identify three different begomoviruses (ACMV, EACMV, EACMV/CM) in cassava field. These viruses are responsible for Cassava Mosaic Disease that is the most devastating disease of Cassava in Africa. For individual virus identification, specific primers were used using the uniplex PCR method. The process used primers JSP001/JSP002, CMBRepF/EACMVRepR, and VNF031/VNF032 for individual identification of ACMV, EACMV and EACMV/CM viruses respectively. This method is very costly and time-consuming for the diagnosis of several viruses in a very large number of samples.

Multiplex PCR has been developed in this study for the simultaneous detection of mixed infections of ACMV, EACMV and EACMV/CM. Different conditions were optimized by using different primers concentrations, annealing temperatures, extension times to achieve optimum conditions for the multiplex PCR. Multiplex PCR enabled for the first time the simultaneous detection of only two CMBs, ACMV and EACMV [12] and it is followed by other research on the detection of more than two viruses [13] [16]. The Mixed infection of ACMV, EACMV [17] [18] [19] and EACMV/CM [11] [20] [21] as revealed in the present study concurs with that of other parts of Africa in which more the one CMB species were reported from CMD-affected cassava plants. On the other hand, these low observed bands could also be explained by the fact that multiplex PCRs using capsid protein-specific primers do not necessarily give better results with a balanced intensity of amplified DNA fragments for each virus compared with multiplex PCRs based on Rep-specific primers [12]. Overall, the results showed that there was no significant difference between simple PCR and multiplex PCR, with both procedures generating amplicons of the targeted product. When the two processes were compared, they are distinct of volume of consumables and number of reactions used. Multiplex PCR used a quarter of consumables used in the uniplex PCR. The number of reactions to detect each of the viruses in uniplex is 114 compared to 38 in multiplex reactions. The intensities of the PCR amplicons in multiplex PCR were relatively weak for each virus while the intensities of the PCR amplicons in uniplex PCR were strong, presumably due to the number of primers used.

It allowed to confirm the results with those of the uniplex PCR and gives similar results. The sensitivity and efficiency of multiplex PCR assays is greatly influenced by a number of factors, including primer concentration, annealing temperatures, as well as competition between the primers [22] [23]. These pro-

tocols minimize sample handling and can markedly reduce the time, labor, and costs of large-scale diagnosis of cassava viruses because this allows to go faster and reduce the use of consumables. The multiplex PCR test developed in this study provides a faster and more efficient way to detect these viruses and could contribute greatly to virus surveillance.

5. Conclusion

Cassava Mosaïc Disease continues to be the major constraint to cassava production and a serious cause of yield reduction of cassava in Togo. The use of multiplex PCR made it possible to detect ACMV, EACMV and EACMV/CM in mixed infections and can also be used for studies of the relation between mixed infections of viruses responsible for CMD in Africa. This method is cost-effective, rapid, sensitive, specific and reliable for the diagnosis of CMB in cassava plants. Also, Multiplex PCR is an excellent diagnostic tool for Cassava mosaic disease surveillance and for disease outbreak prevention or disease effective control.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Tan, S.L (2015) Cassava-Silently, the Tuber Fills: The Lowly Cassava, Regarded as a Poor man's Crop, May Help Save the World from the Curse of Plastic Pollution. *UTAR Agriculture Science Journal*, **1**, 12-24.

[2] Burns, A., Gleadow, R., Cliff, J., Zacarias, A. and Cavagnaro, T (2010) Cassava: The Drought, War and Famine Crop in a Changing World. *Sustainability*, **2**, 3572-3607. https://doi.org/10.3390/su2113572

[3] Ntawuruhunga, P.G., Okao-Okuja, A., Bembe, M., Obambi, J.C., Mvila, A. and Legg, J.P. (2007) Incidence and Severity of Cassava Mosaic Disease in the Republic of Congo. *African Crop Science Journal*, **15**, 1-9. https://doi.org/10.4314/acsj.v15i1.54405

[4] N'zue, B., Zohouri, P.G. and Sangare, A. (2004) Performances agronomiques de quelques variétés de Manioc (Manihot esculenta Crantz) dans trois zones agroclimatiques de la Côte d'Ivoire. *Agronomie Africaine*, **16**, 1-7.

[5] Maruthi, M.N., Colvin, J., Seal S. and Thresh, J.M. (2002) First Report of a Distinct Begomovirus Infecting Cassava from Zanzibar. *Plant Disease*, **86**, 187-187. https://doi.org/10.1094/PDIS.2002.86.2.187A

[6] Fauquet, C.M., Briddon, R.W., Brown, J.K., Moriones, E., Stanley, J., Zerbini, M. and Zhou, X. (2008) Geminivirus Strain Demarcation and Nomenclature. *Archives of Virology*, **153**, 783-821. https://doi.org/10.1007/s00705-008-0037-6

[7] Harrison, B.D., Zhou, X., Otim-Nape, G.W., Lui, Y., Robinson, D.J. (1997) Role of a Novel Type of Double Infection in the Geminivirus Induced Epidemic of Severe Cassava Mosaic in Uganda. *Annals of Applied Biology*, **131**, 437-448. https://doi.org/10.1111/j.1744-7348.1997.tb05171.x

[8] Pita, J.S., Fondong, V.N., Sangare, A., Otim-Nape, G.W., Ogwal, S. and Fauquet, C.M. (2001) Recombination, Pseudo Recombination and Synergism of Geminiviruses are Determinant Keys to the Epidemic of Severe Cassava Mosaic Disease in Uganda. *Journal of General Virology*, **82**, 655-665. https://doi.org/10.1099/0022-1317-82-3-655

[9] Berry, S. and Rey, M.E.C. (2001) Molecular Evidence for Diverse Populations of Cassava Infecting Begomoviruses in Southern Africa. *Archives of Virology*, **146**, 1795-1802. https://doi.org/10.1007/s007050170065

[10] Bull, S.E., Briddon, R.W., Sserubombwe, S., Ngugi, K., Markham, P.G. and Stanley, J. (2006) Genetic Diversity and Phylogeography of Cassava Mosaic Viruses in Kenya. *Journal of General Virology*, **87**, 3053-3065. https://doi.org/10.1099/vir.0.82013-0

[11] Ogbe, F.O., Thottappilly, G., Dixon, A.G.O. and Mignouna, H.D. (2003) Variants of East African Cassava Mosaic virus and Its Distribution in Double Infections with African Cassava Mosaic Virus in Nigeria. *Plant Disease*, **87**, 229-232. https://doi.org/10.1094/PDIS.2003.87.3.229

[12] Alabi, O.J., Kumar, P.L. and Naidu, R.A. (2008) Multiplex PCR Method for the Detection of African Cassava Mosaic Virus and East African Cassava Mosaic Cameroon Virus in Cassava. *Journal of Virological Methods*, **154**, 111-120. https://doi.org/10.1016/j.jviromet.2008.08.008

[13] Abarshi, M.M., Mohammed, I.U., Jeremiah, S.C., Legg, J.P., Lava Kumar, P., Hillocks, R.J. and Maruthi, M.N. (2012) Multiplex RT-PCR Assays for the Simultaneous Detection of Both RNA and DNA Viruses Infecting Cassava and the Common Occurrence of Mixed Infections by Two Cassava Brown Streak Viruses in East Africa. *Journal of Virological Methods*, **179**, 176-184. https://doi.org/10.1016/j.jviromet.2011.10.020

[14] Elnifro, E.M., Ashshi, A.M., Cooper, R.J. and Klapper, P.E. (2000) Multiplex PCR: Optimization and Application in Diagnostic Virology. *Clinical Microbiology Reviews*, **13**, 559-570. https://doi.org/10.1128/CMR.13.4.559

[15] Fondong, V.N., Pita, J.S., Rey, M.E.C., De Kochko, A., Beachy, R.N. and Fauquet, C.M (2000) Evidence of Synergism between African Cassava Mosaic Virus and a New Double-Recombinant Geminivirus Infecting Cassava in Cameroon. *Journal of General Virology*, **81**, 287-297. https://doi.org/10.1099/0022-1317-81-1-287

[16] Aloyce, R.C., Tairo, F., Sseruwagi, P., Rey, M.E.C. and Ndunguru, J. (2013) A Single-Tube Duplex and Multiplex PCR for Simultaneous Detection of Four Cassava Mosaic Begomovirus Species in Cassava Plants. *Journal of Virological Methods*, **189**, 148-156. https://doi.org/10.1016/j.jviromet.2012.10.007

[17] Adjata, K.D., Muller, E., Peterschmitt, M., Aziadekey, M. and Gumedzoe, Y.M.D (2010) Incidence of Cassava Viral Diseases ANS First Identification of East African Cassava Mosaic Virus and Indian Cassava Mosaic Virus by PCR in Cassava (Manihot Exculenta Crantz) Fields in Togo. *American Journal of plant Physiology*, **5**, 94-101. https://doi.org/10.3923/ajpp.2008.73.80

[18] Busgoro, J.P., Masquellier, L., Kummert, J., Dutrecq, O., Lepoivre, P. and Jijakli, M. (2008) Application of a Simplified Molecular Protocol to Reveal Mixed Infections with Begomoviruses in Cassava. *Journal of Phytopathology*, **156**, 452-457. https://doi.org/10.1111/j.1439-0434.2008.01391.x

[19] Torkpo, S.K., Offei, K., Danquah, E.Y., and Gafni, Y (2017) Status of Cassava Mosaic Begomoviruses in Farmers' Fields in Ghana. *AIMS Agriculture and Food*, **2**, 279-289. https://doi/org/10.3934/agrfood.2017.3.279

[20] Soro, M., Tiendrébéogo, F., Pita, J.S., Traoré, E.T., Somé, K., Tibiri, E.B., James B., Néya J., Mutuku, M., Simporé, J. And Koné, D. (2021) Epidemiological Assessment of Cassava Mosaic Disease in Burkina Faso. *Plant Pathology*, **70**, 2207-2216. https://doi.org/10.1111/ppa.13459

[21] Anitha, S., Monyo, E.S. and Okori, P. (2014) Simultaneous Detection of Groundnut Rosette Assistor Virus (GRAV), Groundnut Rosette Virus (GRV) and Satellite RNA (satRNA) in Groundnuts Using Multiplex RT-PCR. *Archives of Virology*, **159**, 3059-3062. https://doi.org/10.1007/s00705-014-2139-7

[22] Chou, Q., Russel, M., Birch, D.E., Raymond, B.J. and Bloch, W (1992) Prevention of Pre-PCR Mis-Priming and Primer Dimerization Improves Low-Copy-Number Amplification. *Nucleic Acids Research*, **11**, 1717-1723. https://doi.org/10.1093/nar/20.7.1717

[23] Wei, T., Lu, G. and Clover, G.R.G (2009) A Multiplex RT-PCR for the Detection of Potato Yellow Vein Virus, Tobacco Rattle Virus and Tomato Infectious Chlorosis Virus in Potato with a Plant Internal Amplification Control. *Plant Pathology*, **58**, 203-209. https://doi.org/10.1111/j.1365-3059.2008.01979.x