Med. Fac. Landbouww. Univ. Gent, 63/4b, 1998

DEVELOPMENT OF ROUTINE DETECTION TESTS USING PCR FOR CERTIFICATION

A. Chandelier, S. Cognet, V.L. Marinho, J. Kummert and P. Lepoivre
Unité de Phytopathologie, Département de Biochimie et Biologie Appliquées
Faculté Universitaire des Sciences Agronomiques, Passage des Déportés, 2
B-5030 Gembloux, Belgium

SUMMARY

Current developments in agricultural and horticultural production systems require the availability of rapid, sensitive, reliable and user's friendly detection protocols applicable for routine testing in certification procedures. Although the polymerase chain reaction (PCR) allows to reach the demands for sensitivity, it usually requires the use of purified nucleic acids preparations as template, which hampered the routine use of the PCR technology on a large number of samples. Moreover, for the amplification from RNA sequences, an independant preliminary reverse transcription (RT) step has to be performed. Another drawback in the use of PCR protocols, as usually applied, is the relative lack of sensitivity in the detection of the amplification products by UV illumination after electrophoresis in ethidium bromide stained agarose gels. The simplification of the (RT)-PCR protocols for routine detection has thus to meet different requirements : (1) the reduction of the risk of contaminations linked to the manipulation of large samples, (2) the simplification of sample preparation, and (3) the use of alternative detection protocols giving more specificity, sensitivity and performances facilities.

In our laboratory, several alternatives to classical extraction of nucleic acids, including silica capture (for bacteria and viruses), immunocapture or the direct use of diluted crude sap (for viruses) have been successfully used for the PCR detection of targeted sequences. Furthermore, a colorimetric detection of the PCR products in microtitration plates has been developed for all the pathogens studied. For the detection of bacteria (Erwinia carotovora and Ralstonia solanacearum) and potato viruses, the amplicons are revealed after sandwich hybridization between a covalently linked capture probe and a biotinylated detection probe (Lambdatech s.a., kits in development). For fruit trees, either DIG-labeled amplicons, or whenever possible amplification products hybridized to specific DIG-labeled probes, are fixed to a streptavidin coated microplate by means of a biotin labeled capture probe.

These assays, combining the sensitivity and the specificity of the PCR but also the easiness of both the sample preparation and the detection of the amplicons, can be partly automated. They offer the possibility to analyse a great deal of samples and are thus well adapted for routine testing in the frame of quality control programme of planting material.

INTRODUCTION

Phytosanitary certification of planting material is becoming compulsory for many crops, strengthening the necessity of developing sensitive and reliable tests adapted to the characteristics of the pathogen (threshold of inoculum, variability,...). In this context, a diagnostic technique must give the guarantee of adequate sensitivity and specificity (in order to confine to a small and

predescribed range the probabilities of both false positive and false negative results). The test must be also practicable for routine application on a large number of samples.

Although PCR has reached wide acceptance in research laboratories for the diagnosis of plant pathogens (Henson and French, 1993), difficulties linked to the pre- and post-amplification steps represent major drawbacks for its application in routine tests. Indeed, samples preparation usually requires cumbersome and expensive protocols. Moreover, although the detection of the amplified products by electrophoresis in ethidium bromide stained agarose gels has proven to be easy to perform, that technique suffers from its lack of both sensitivity and specificity and can not be automated.

In an effort to improve the suitability of PCR technology in a context of certification programs, pre- and post-PCR protocols adapted to the analysis of a great deal of samples are progressively available.

The present communication focuses on the different steps which must be taken into account in the conception of a rapid, cost-effective and efficient detection kit based on PCR. These different steps are illustrated with original results obtained in our labortory.

THE DIFFERENT STEPS IN THE CONCEPTION OF A DETECTION TEST BASED ON PCR

1. PCR assay optimization : the selection of primers

Among the different parameters which have to be optimized in PCR assays (PCR mixture, temperature cycling regime, enzyme used...), appropriate primers selection has a major importance for successful detection of plant pathogens as it will greatly influence the reliability and robustness of the test and govern its specificity. Primers have to meet different requirements in order to avoid false positive or false negative results.

The first step is to select adequate target sequences, according to the desired specificity of the test (strain, species, genus,..). Indeed, amplifications performed with primers which do not target the adequate genomic sequences can give rise to erroneous results. As an example, false negative results are frequent when the primers selection rests on the sequence of one single strain of a pathogen without paying attention to the variability existing between isolates. On the other hand, artifactual products can be generated by a too low annealing temperature, which is adopted for maintaining an acceptable level of sensitivity when primers do not perfectly match their targeted sequences (i.e. degenerate primers or primers with mismatched nucleotides).

Besides the constraints linked to the biological objectives, the test efficiency will also be influenced by technical constraints linked to the primer sequence. In that respect, computer programs able to analyse the design of PCR primers, but also to simulate a PCR reaction are very helpful as they take into account constraints linked to the primers (3'clamp, sequence ambiguity, self-annealing, difference in primers Tm...) but also other constraints linked to the amplified product (length, GC content).

2. The samples processing protocol

The samples processing protocols should be adapted to the analysis of a great deal of samples and compatible with an efficient amplification step. Generally, people using the PCR technology are working in research laboratories and use or develop efficient nucleic acid purification protocols which are nevertheless time-consuming or too expensive for use in routine tests.

In this view, the use of crude plant extract represents the ultimate simplification of the PCR assay. However, as plant tissues often contain components interfering with the PCR reaction (Rowhani *et al*, 1993), the crude extract has to be sufficiently diluted, thus making necessary the use of well designed primers in order to compensate the decrease in template concentration by a performent amplification step. Two detection kits which are in development in our laboratory use diluted crude sap as a target. In spite of the dilution required by the sample processing procedure, these kits appear sensitive enough to detect latent infections. For the detection of potato virus Y (PVY), a RT-PCR protocol allows to detect PVY in dormant tubers using diluted crude sap, without any preliminary purification of the template. For the detection of Apple Stem Groving Virus (ASGV) in apple trees, a RT-PCR protocol using diluted crude sap from leaves or bark tissues (Marinho *et al*, in press) is able to detect the virus in all seasons (in contrast to ELISA tests).

Immunocapture of the pathogen in the crude extract prior to the PCR assay represents a first alternative if the use of crude sap is not directly possible. Such protocols combining immunocapture and PCR have been successfully used for the detection of plant pathogens (Wetzel *et al*, 1992; Nolasco *et al*, 1993). In our laboratory, this technology is applied for the detection of *Ralstonia solanacearum* in potato tubers, and the Apple Chlorotic Leaf Spot Virus (ACLSV) in apple trees. As we did not succeed in amplifying DNA molecules from diluted crude sap for the detection of *R. Solanacearum*, an immunomagnetic separation protocol using beads coated with sheep anti-rabbit IgG and a polyclonal serum raised against *R. solanacearum* [kindly provided by Dr Van der Wolf (IPO-DLO)] was adapted from a protocol published for *Erwinia carotovora* subsp. *atroseptica* (Van der Wolf *et al*, 1996). For the detection of ACLSV, an immunocapture RT-PCR protocol has been developped using anti-ACLSV IgG (Kummert *et al*, 1995).

And last but not least, nucleic acids extraction protocols adapted to routine testing are progressively reported and commercial kits for plant nucleic acid extraction are now available.These kits, however, are quite expensive and may prove unsuitable for large scale applications.

3. The detection of amplification products

Post-PCR product detection is generally carried out by electrophoresis in ethidium bromide stained gels. This technique is rapid and easy to perform in research laboratory with a small number of samples. However, as it can not be automated and use a very toxic molecule, it is not adapted for large scale analysis. Furthermore, this detection method is not very sensitive, giving rise to frequent false negative results, and does not discard false positive results because its specificity rests only on the size of the amplification products.

Detection of the PCR products by hybridization assays using non-radioactive specific probe in a diagnosis format adapted to routine testing is a prerequisite for routine tests. Different protocols based on such approach have been proposed. The "*taq*man" system, developed by Roche Molecular Systems, Inc., has been successfully used in clinical tests, notably for the detection of human papillomavirus (Swan *et al*, 1997) and also more rarely for the detection of plant pathogens (Schoen *et al*, 1996). A fluorogenic probe, consisting in an oligonucleotide with a 5' reporter and a 3' quencher dyes attached at its extremities, specifically hybridizes to the PCR product. During each extension cycle, this probe is cleaved by the 5'nuclease activity of the *taq*DNA polymerase, thus releasing the reporter dye and giving rise to fluorescence. Molecular beacons, i.e. probes which fluoresce upon hybridization (Tyagi *et al*, 1996), have also been described for the detection of NASBA-amplified products (Leone *et al*, 1998). In this case, the probes, which could be used for the detection of PCR products, form an hairpin structure when not hybridized. A reporter and a quencher dyes are attached to each hairpin extremity. When such probes hybridize to their targeted sequences, the hairpin structures are disrupted thus leading to fluorescent signals. Such systems

using fluorescence are very attractive as the detection of the amplicons is performed in real-time, without the need of post-PCR operation. However, these techniques require fluorometers whose cost represents a major drawback for plant certification.

Our laboratory adopted a colorimetric detection of amplicons in microtitration plates. This protocol rests on a specific sandwich hybridization of the amplified products between a well-linked capture probe and a labeled detection probe (ELOSA test). This design presents several advantages : 1°/ it allows the analysis of a great deal of samples in an ELISA format; 2°/ both the capture probe and the detection probe contribute to ensure a high specificity for the detection, thus decreasing the risk of false positive results; 3°/ this detection method is from 10 times to 100 times more sensitive than analysis of stained agarose gel according to the pathogen tested; 4°/ in contrast to the above mentioned methods detecting fluorescent signals, the widely used ELISA equipment (i.e. spectrophotometer) may be used for the detection of the PCR products; 5°/ for large-scale analysis in automated laboratories, it is possible to combine the PCR assay and the sandwich hybridization in an ELISA format using devices which will perform all the operations, from the PCR amplification to the amplicon detection, in an automated way.

We developed two designs of sandwich hybridization according to the pathogen detected. For fruit tree viruses, the amplification products are fixed to a streptavidin coated plate by means of a biotynilated capture probe. The detection is performed using an internal specific DIG-labeled probe. This design has proven to be specific and sensitive. A second design used for the detection of bacteria (*Erwinia carotovora* and *Ralstonia solanacearum*) and virus (PVY) in potato tubers has been elaborated in collaboration with the private company Lambdatech s.a. (Belgium). This second design, consisting in an hybridization of the amplification products between a covalently-linked capture probe and a biotynilated detection probe, has proven to be as specific and sensitive than the first one but appears more rapid and less expensive to perform.

CONCLUSION

From an economical point of view, PCR is still in its infancy for large scale diagnosis of plant pathogens. Cost is a crucial parameter in the development of this technology. As labour cost represents a significative part of the total cost, private companies are working on an automation of the procedure. The simplification of the samples preparation is another challenge. Different solutions from the simplest one to more elaborated protocols are proposed.

The conception of a PCR assay should integrate in an overall way the three steps of the procedure, i.e. the sample preparation, the amplification step (mainly selection of primers) and the detection of the amplicons. A correct design of primers will generally enable to simplify samples processing. Moreover, in spite of technical constraints linked to the PCR reaction, the amplicon characteristics will also have to be compatible with the detection system chosen. Some criteria like the PCR product length or the presence of specific internal hybridization sequences have to be taken into account when selecting the primers.

Finally, we must take into account that the reliability of a quality control of plant material depends on both the characteristics of the tests and the sampling protocol which may also have an impact on the cost of the test. The sampling methods which are used in reference tests (generally ELISA) are not necessarily adapted to diagnostic tests based on PCR. They should thus be reevaluated and maybe modified taken into account both the characteristics (mainly sensitivity and cost) of the PCR technology and the phytosanitary thresholds to be certified.

Acknowledgments :

This communication presents the results of different research programmes supported by (i) the "Ministère (fédéral) des Classes Moyennes et de l'Agriculture – DG6" Brussels, (ii) the "Direction Générale des Technologies, de la Recherche et de l'Energie de la Région Wallonne" Namur, Belgium and (iii) the EEC (contract FAIR-5 PL3889).

BIBLIOGRAPHY

Henson JM, French R (1993) The polymerase chain reaction and plant diagnosis. Annu. Rev. Phytopathol 31, 81 – 109

Kummert J, Rufflard G, Colinet D, Lepoivre P (1995) Detection of apple chlorotic leaf spot virus and apple stem groving virus by RT-PCR and IC-RT-PCR. Med. Fac. Landbouww. Univ. Gent 60/2a, 277-281

Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD (1998) Molecular beacons probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. Nucleic Acids Res. 26, 2150-2155

MarinhoVLA, Kummert J, Rufflard G, Colinet D, Lepoivre P (1998) – Detection of apple stem groving virus in dormant apple trees with crude extract as templates for one-step RT-PCR. Plant Dis., (in press)

Nolasco G, de blas C, Torres V, Ponz F (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. J. Virol. Methods 45, 201-218

Rowhani A, Chay C, Golino DA, Falk BW (1993) Development of a polymerase chain reaction technique for the detection of grapevine fanleaf virus in grapevine tissue. Phytopathology 83, 749-753

Schoen CD, Knorr D, Leone G (1996) Detection of potato leafroll virus in dormant potato tubers by immunocapture and a fluorogenic 5'nuclease RT-PCR assay. Phytopathology 86, 993-999

Swan DC, Tucker RA, Holloway BP, Icenogle JP (1997) A sensitive, type-specific, fluorogenic probe assay for detection of human papillomavirus DNA. J. Clin. Microb. 35, 886-891

Tyagi S, Kramer FR (1996) Molecular Beacons : Probes that fluoresce upon hybridization. Nature Biotechnology 14, 303-308

Van der Wolf JM, Hyman LJ, Jones DAC, Grevesse C, van Beckhoven JRCM, van Vuurde JWL, Pérombelon MCM (1996) Immunomagnetic separation of *Erwinia carotovora* subsp. *atroseptica* from potato peel extracts to improve detection sensitivity on a crystal violet medium or by PCR. J. Appl. Bact. 80, 487-495

Wetzel T, Candresse T, Macquaire G, Ravelonandro M, Dunez J (1992) – A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. J Virol Methods 39, 27-37