Preliminary studies on the use of the Cascade Rolling Circle Amplification technique for *Plum pox virus* detection

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Abstract

Isothermal techniques for the amplification of nucleic acids have emerged in the last years. In contrast to the Polymerase chain reaction (PCR), the most prevalent method to amplify DNA *in vitro*, the reactions can be run at constant temperatures. Specificity and sensitivity are at least as high as that obtained by using PCR and the methods are less time consuming. Therefore, the isothermal amplification of nucleic acids provides a powerful tool for the detection of *Plum pox virus* (PPV), the causal agent of the Sharka disease.

The cascade rolling circle amplification (CRCA), first described by Thomas et al. (1999), is based on the rolling circle mechanism that many viruses use to replicate their genome multiplicatively. Circular Probes, also called Padlock probes (PLP), which arise from the ligation of the terminal region of DNA probes upon side by side hybridization to the target serve as template (Nilsson et al. 1994).

For detecting PPV by CRCA, RNA was extracted and reverse transcribed to cDNA using a PPV specific primer. Several PLPs with varying lengths and sequences complementary region to the cDNA were designed and tested. Furthermore, different pairs of primers for the subsequent amplification were developed. For specific ligation Ampligase and T4 DNA Ligase were tested. In CRCA, two polymerases with strong strand displacement activity were compared: Phi29 DNA Polymerase and *Bst* DNA Polymerase. These enzymes differ in their optimal reaction temperature.

Ligation as well as amplification do occur, but there is high background amplification also in negative and no template controls. Discrimination was possible after a restriction digestion is carried out. As proven by sequencing of reaction products non-specific signals were a result of primer polymerization. Current work focuses on the reduction of the background amplification and improvement of the sensitivity.

Keywords: Cascade Rolling Circle Amplification, CRCA, isothermal amplification of DNA, *Plum pox virus*, *PPV*

Introduction:

Today the Polymerase chain reaction (PCR) is the tool of choice to amplify DNA *in vitro* because of its versatile capabilities in the field of molecular biological research. However, in recent years several isothermal techniques for the amplification of nucleic acids have emerged (Gill and Ghaemi 2008). In PCR, denaturation, priming and elongation are done step-by-step at alternating temperatures whereas in isothermal techniques all steps take place simultaneously avoiding the use of a thermal cycler. Regarding specificity and sensitivity these methods are equal to or even better than PCR. Therefore, the isothermal amplification of nucleic acids should also provide a reliable detection method for *Plum pox virus* (PPV), the causal agent of Sharka disease.

Artificial DNA circles with far less than 100 nucleotides can be enzymatically amplified via the same rolling circle mechanism many viruses use to multiply their genome (Fire et al. 1995, Liu et al. 1996). Such a DNA circle, also called Padlock probe (PLP), can arise from the ligation of the terminal regions of a linear DNA probe upon side by side hybridization to the target (Nilsson et al. 1994). The ligase mediated generation of PLPs combined with rolling circle replication represents a powerful system for the detection of DNA with both high specificity and sensitivity (Banér et al. 1998, Lizardi et al. 1998).

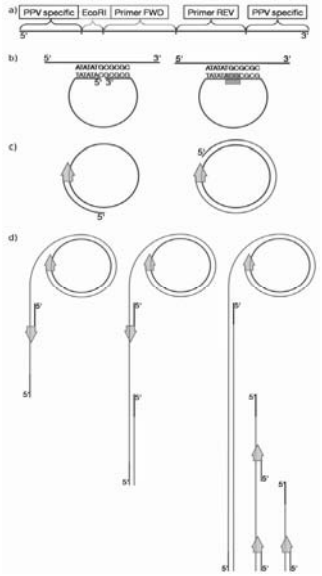

Fig. 1 Cascade rolling circle amplification (CRCA): a) design of a linear DNA probe; b) enzymatic ligation of the PLP upon hybridization to PPV cDNA; c) rolling circle amplification of the circular PLP using a polymerase with strand displacement activity; d) advanced, cascade like amplification.

Thomas et al. (1999) advanced the method with the amplification of the released strand to achieve exponential accumulation of DNA creating the cascade rolling circle amplification (CRCA) (Fig. 1). After gel electrophoresis a ladder like pattern is visible with each step indicating a multimere of the PLP. To verify the use of CRCA for PPV diagnostics preliminary tests were conducted.

Material and methods

For the evaluation of the new PPV detection method based on CRCA PPV free and PPV infected trees of the *Prunus domestica* cultivars `Katinka´ and `Jojo´ were chosen for PPV positive and negative controls, respectively.

RNA was extracted from plum leaves using "Concert™ Plant RNA Reagent" (Invitrogen) according to the manufacturer´s instructions. For the cDNA synthesis carried out by M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) 1 µg total RNA, serving as template, and 25 mM PPV specific primer were filled up to a volume of 17.75 µl by RNase free water and preincubated for 5 min at 70 °C. 5 µl M-MLV reaction buffer, 0.5 mM dNTPs and 200 U of the enzyme were added to obtain a final volume of 25 µl. The incubation time was 1 h starting at 40 °C and raising the temperature to 48 °C after 10 min.

For general detection of PPV the sequences of several isolates representing all PPV subgroups known so far were aligned using ClustalW as hosted on http://www.ebi.ac.uk/Tools/clustalw/index.html. A homologous region located in the coding sequence of the coat protein was selected as the hybridization site of the PLP to the cDNA (Fig. 3).

The PLP consists of two complementary sequences at the terminal regions linked with the restriction site of *EcoR*I, the complementary sequence of Primer FWD and the identical sequence of Primer REV (Fig. 1). These primers were designed to bind exclusively to the PLP and its complement respectively, but not to PPV and known *Prunus* nucleotide sequences as screened by BLAST analysis. 0.5 U Ampligase® Thermostable DNA Ligase (Epicentre™ Biotechnologies) was used to join the 5'- and the 3'-end of the linear PLP after hybridization to PPV cDNA at 64 °C for 15 minutes with three minutes of preincubation at 94 °C in a volume of 15 µl containing Reaction Buffer, 0.12 µM PLP and cDNA. After adding ThermoPol Buffer, 0.2 µM dNTPs, 1 µM of each primer, 0.75 M betaine and 1.6 U *Bst* DNA Polymerase (New England Biolabs (NEB)) to an aliquot of the ligation reaction the CRCA was carried out in 60 minutes at 65 °C. The enzyme was inactivated by heat (20 min at 80 °C). For restriction digestion, *EcoR*I (Fermentas) was used.

Comparison of Ligases: There are a number of different ligases available. To test which DNA joining enzyme provides the optimal basis for the following amplification three further ligases were compared with the standard protocol mentioned above: *Taq* DNA Ligase (New England Biolabs) and 9°N™ DNA Ligase (New England Biolabs) are active at elevated temperatures like Ampligase® Thermostable DNA Ligase, whereas T4 DNA Ligase (New England Biolabs) has its optimum at 37 °C. Results obtained by *Taq* DNA ligase were similar to those by Ampligase® Thermostable DNA Ligase: strong amplification of the positive control, but also background amplification in negative and no template control, so definite discrimination was only possible after restriction digestion. 9°N™ DNA Ligase resulted in a very weak signal. The T4 DNA Ligase produced DNA circles in either case at the same rate indicating an unspecific reaction as a result of the ligation of the PLP without hybridisation to a target (Fig. 2a).

Activity of Polymerases: The second type of enzyme used in this method is the polymerase. The range of polymerases that meet the demand of high strand displacement activity is limited. *Bst* DNA Polymerase was tested against IsoTherm™ DNA Polymerase (Epicentre™ Biotechnologies), DisplaceAce™ DNA Polymerase (Epicentre™ Biotechnologies), both active at 65 °C, and Phi29 DNA Polymerase (New England Biolabs), which exhibits a very high processivity with an optimum at 37 °C. The CRCA performed by the *Bst* DNA Polymerase yielded high amounts of DNA. The incubation time in case of IsoTherm™ and DisplaceAce™ DNA Polymerase was twice as long as that of *Bst* DNA Polymerase, but the intensity of the signal using these thermophilic enzymes was weaker. Phi29 DNA Polymerase did not synthesize any DNA even when using exonuclease resistant primers (Fig. 2b).

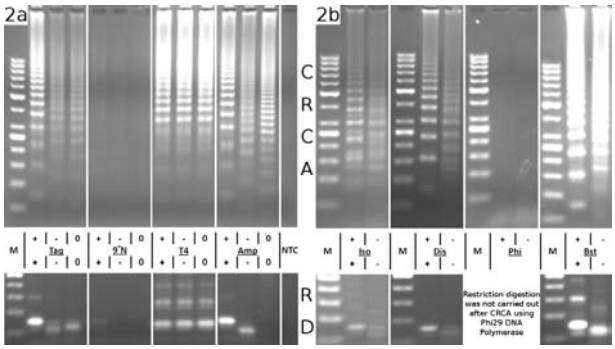

Fig. 2 **a**: CRCA of a DNA probe PLP4 ligated by four different ligases. Taq: *Taq* DNA Ligase, 9°N: 9°N™ DNA Ligase, T4: T4 DNA Ligase, Amp: Ampligase® Thermostable DNA Ligase; +: positive control, -: negative control, 0: no template control, NTC: control without ligation reaction, M: GeneRuler™ 50bp DNA Ladder**;** CRCA: cascade rolling circle amplification, RD: restriction digestion. **2b**: CRCA of the DNA probe PLP4 with four DNA polymerases. Iso: IsoTherm™ DNA Polymerase, Dis: DisplaceAce™ DNA Polymerase, Phi: Phi29 DNA Polymerase, Bst: *Bst* DNA Polymerase; +: positive control, -: negative control, M: GeneRuler™ 50bp DNA Ladder**;** CRCA: cascade rolling circle amplification, RD: restriction digestion.

Probe and Primer Design: Both the primer sequence and the PPV complementary regions of the PLP were varied because of the potential for a high background signal . All designed primers were specific just to the PLP and not to plum or PPV. As proven by the sequencing of reaction products non-specific signals are a result of primer polymerization, which do also occur with two other pairs of primer. Moving the hybridization region (Fig. 3) on the PPV cDNA resulted in a lower signal strength, whereas the variation of the length of the PPV complementary region caused hardly any alternation but did not change the intensity of the background signal either.

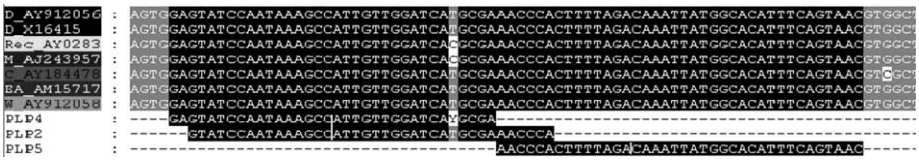

Fig. 3 Alignment of the nucleotide sequence of seven PPV isolates representing all known PPV subgroups and the recognition sites of the tested DNA probes (PLP). Gene bank accession numbers are listed in the picture. The yellow bar indicates the ligation site.

Conclusion

Ligation as well as amplification did occur, but there was high background amplification also in negative and no template controls. Discrimination is possible after restriction digestion is carried out. For establishing a reliable and sensitive PPV detection method the background amplification must be reduced and sensitivity increased.

Acknowledgements

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