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## Optimization of cDNA Amplification of Apricot Latent Virus (ApLV) from Various Plant Tissues Sources

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**Abstract:** Although the reverse transcriptase polymerase chain reaction (RT-PCR) procedure is basically simple operation, often it is not possible to achieve optimum results without optimizing the protocols. An RT-PCR method targeting a 200 bp sequence of the CP gene of Apricot Latent Virus (ApLV) was used as a model to improve the detection limit and to compare the behavior of three different plant tissues in a RT-PCR assay. A number of factors should be considered when selecting the optimal system for RT-PCR. Important considerations include the optimal concentrations of MgCl<sub>2</sub>, dNTP, *Taq* DNA polymerase enzyme, specific primer and the amount of cDNA for the downstream applications. This study therefore discusses a series of critical PCR parameters and feasible strategies for optimization of RT-PCR detection of ApLV.

**Key words:** ApLV, RT-PCR, optimization

### INTRODUCTION

Since its introduction, the polymerase chain reaction has found a solid niche in broad areas of agriculture, industrial, forensic and medical communities where people routinely handle nucleic acids. Applications in these areas have developed mainly as a result of PCR's high sensitivity (Park and Lee, 2003). A critical step in developing a new PCR assay is to optimize the reaction conditions to obtain maximum specificity and sensitivity. The primary reason for these optimization steps is determine what deviations from the standard reaction conditions are necessary to promote functional primer annealing and extension (Miesfeld, 1999). Amplification efficiency is influenced by a number of factors including target length and sequence, primer sequence, buffer conditions, sample impurities, cycling conditions and PCR enzyme. Since PCR consists of many amplification steps, adjusting reaction conditions to achieve even slight improvements in amplification efficiency can lead to dramatic increases in PCR product yield (Arezi *et al.*, 2003).

Although PCR is now a routine technique in many laboratories, there are still a considerable number of problems in getting good, reproducible amplifications (Seal and Cotes, 1998). Unquestionably, no single protocol will be appropriate to all situations (Innis and Gelfand, 1990). In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume (Usta *et al.*, 2005).

Apricot Latent Virus (ApLV) is a definitive species in the genus *Foveavirus*, family *Flexiviridae* (Adams *et al.*, 2004). The virus was first described in Moldova from latently infected apricot (*Prunus armeniaca*) cv. Silistra introduced from Bulgarian (Zemtchik and Verderevskaya, 1993; Zemtchik *et al.*, 1998). The virus naturally infects apricot trees with no apparent symptoms. ApLV however, cause yellow spot symptom on leaves of graft-inoculated peach seedlings (Zemtchik and Verderevskaya, 1993; Zemtchik *et al.*, 1998). The virus was reported in France, Italy (Gentit *et al.*, 2001), Palestine (Abou Ghanem-Sabanadzovic *et al.*, 2002) and more recently in Turkey (Gumus *et al.*, 2004).

Recently, increasing interest and efforts from a variety of approaches have been directed to the quality assurance and standardization of the PCR technique (Yang *et al.*, 2004). PCR is a technique for genetic screening, detection studies and microsatellite applications where it is necessary to amplify specific products in a single or double reaction. The technique often requires extensive optimization because of nonspecific products, which may interfere with the amplification process. Although the mis-annealing temperatures can usually be avoided by using gradient functioned PCR cyclers, amplification specificity is also influenced by other factors such as primer, enzyme, dNTP and Mg<sup>2+</sup> ion concentration. In this study therefore we describe an optimization strategy for the detection of ApLV from different plant tissue sources.

## MATERIALS AND METHODS

**Plant and virus source:** An apricot (cv. Tyrinthe) isolate of ApLV was used as a reference isolate. The virus was transmitted to a healthy apricot (*Prunus armeniaca* L.) seedling and maintained outside. Inoculated plant was served as a virus source during the trials. Leaf, one-year old shoots and bark tissues of systemically infected *P. armeniaca* was used in RT-PCR assays.

**RNA extraction and reverse transcription:** Total RNA was recovered with silica-based method according to study made by Fossiac *et al.* (2000) with slight modifications. Approximately 100 mg of infected fresh leaf, bark and one year old shoot tissues were homogenized with 2 mL of grinding buffer containing 4.0 M Guanidine thiocyanate, 0.2 M NaOAc, 25 mM EDTA, 1.0 KOAc, 2.5% w/v PVP-40, 1% 2-mercapto ethanol. Aliquots of 500  $\mu$ L of extracts were mixed with 100  $\mu$ L of 10% sodium lauryl sarcosyl solution in a new set of sterile eppendorf. Tubes were incubated at 70°C with intermittent shaking for 10 min and incubated in ice for 5 min. After centrifugation at 14,000 rpm for 10 min, 300  $\mu$ L of the supernatant was transferred to a new eppendorf set containing 150  $\mu$ L of ethanol, 25  $\mu$ L of resuspended silica and 300  $\mu$ L of 6 M sodium iodide. The mixture was then incubated at room temperature for 10 min with intermittent shaking. After centrifugation at 6,000 rpm for 1 min, supernatant discarded and the pellet washed twice with washing buffer. The pellet then was resuspended with 150  $\mu$ L of RNase-free water and incubated for 4 min at 70°C followed by a centrifugation at 14,000 rpm for 3 min. Finally the supernatant was transferred to a new eppendorf set and stored at -20°C until use. Oligonucleotide primer sequences (ApLV1 5'-GGAATAGAGCCCCAAGAAG-3' and ApLV2 5'-AGCAAGGTAAACGCCAAC-3') reported by Nemchinov and Hadidi (1998) were used to detect ApLV. The amplified fragment was in length of 200 bp. The sequence of reverse complementary primer (ApLV2) was used for viral cDNA synthesis (Nemchinov and Hadidi, 1998).

**PCR amplification of cDNA:** One micro liter of cDNA was mixed with 24  $\mu$ L of the amplification mixture containing 2.5  $\mu$ L of the 10X reaction buffer (200 mM Tris-HCL pH:8.4, 500 mM KCl) 1.5  $\mu$ L of MgCl<sub>2</sub> (25 mM), 0.5  $\mu$ L dNTPs (10 mM), 0.5  $\mu$ L of each primer (100 pmol  $\mu$ L<sup>-1</sup>), 0.2  $\mu$ L of *Taq* DNA polymerase (Fermentas) and 17.8  $\mu$ L of RNase free sterile water. Initial denaturation was at 94°C for 1.5 min and followed by 30 cycles of denaturation at 94°C for 20 sec, annealing at 58°C for 30 sec, 72°C for 45 sec and a final extension at 72°C for 5 min 10  $\mu$ L of

amplified PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining. While one of the PCR parameters was increased stepwise the others were kept constant in the optimization of PCR parameters.

## RESULTS

For better detection of ApLV by RT-PCR a number of critical PCR parameters for optimization were investigated. Equivalent concentrations of four dNTPs were used through the trials. In all tried dNTP concentrations ranging from 0.001 to 10 mM expected size of fragments were obtained from all tested tissues (Fig. 1). The best results were at 10 mM of each dNTP. No significant differences were recorded between low and higher concentration of dNTP.

The amount of cDNA was significantly influenced the PCR products. The quantity of amplified fragments was increased when the cDNA amount was gradually increased. As seen in Fig. 2 at cDNA template quantities between 0.2-4/50  $\mu$ L a slight difference was observed, however, below 0.2  $\mu$ L the amount of the products were decreased. Thus, the optimum cDNA amount was ranged between 0.2-4  $\mu$ L for 50  $\mu$ L reaction mixture (Fig. 1).

The concentration of MgCl<sub>2</sub> was significantly influenced the PCR products. As seen in Fig. 2 at first and second lanes, the too low MgCl<sub>2</sub> concentrations

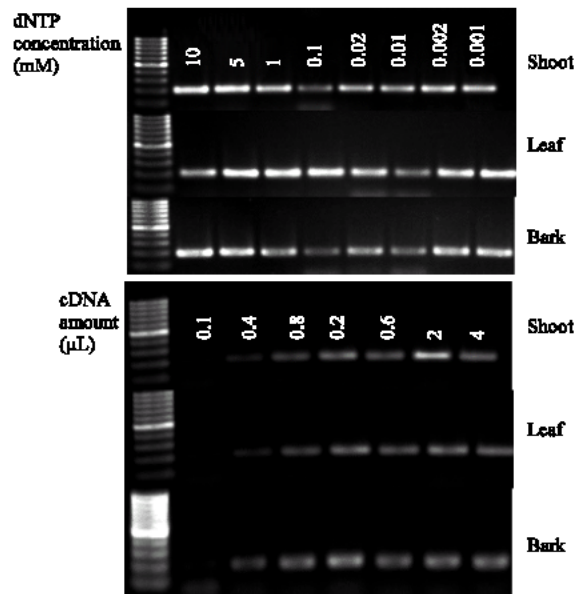


Fig. 1: Electrophoretic analysis of RT-PCR products deriving from different concentration of dNTP and the amount of cDNA template. M, molecular size markers

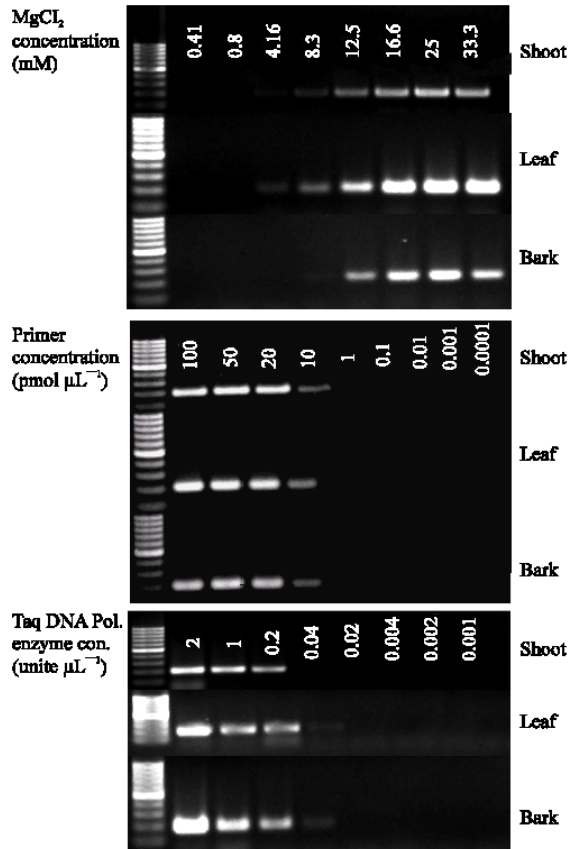


Fig. 2: Electrophoretic analysis of RT-PCR products deriving from different  $MgCl_2$ , primer and *Taq* DNA polymerase enzyme concentrations. M, molecular size markers

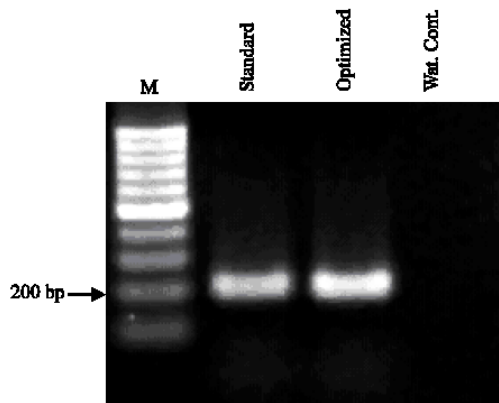


Fig. 3: Simultaneous amplification of ApLV genome with the standard and optimized PCR parameters, Wat. Cont.: water control, M: molecular size markers

were resulted insufficient amount of desired products. The optimum  $MgCl_2$  concentration was ranged between 12-33 mM.

Table 1: Optimum concentration or value of PCR parameters obtained throughout the RT-PCR reactions

Main parameters	Concentrations/amounts	
	Standard	Optimized
dNTP	10 mM	0.001 mM
primers	100 pMol	20 pMol
$MgCl_2$	25 mM	12.5 mM
cDNA	2 $\mu$ L	1.2 $\mu$ L
<i>Taq</i> DNA Pol. enzyme	2 unite	0.2 unite

As shown in Fig. 2, the use of ApLV genome specific primers with the concentration of  $20 \text{ pmol } \mu\text{L}^{-1}$  resulted in the detection of a specific PCR product of the correct size, whereas no reliable signal was obtained from further dilutions of genome-specific primers. The quantity of amplified fragments was decreased when the primer concentration was gradually decreased. Thus, the optimum primer concentration was ranged between 20-100  $\text{pmol } \mu\text{L}^{-1}$ .

Different concentrations of *Taq* DNA Polymerase (Fermentas) were tested using genome specific primers. The most efficient enzyme concentration seemed to be around 0.2-2 unite/ $50 \mu\text{L}$  reaction volume (Fig. 2). Too low enzyme resulted in insufficient amplification products. Although, initially, the amplification rates of *Taq* polymerase and primer dilutions reached the theoretical limit during applied cycles, a sharp decline in amplification rates was observed at further dilutions (Fig. 2).

To determine the sensitivity of the RT-PCR method, standard and optimized concentrations and/or values were used in simultaneous amplification, using standard and optimized concentrations of primer, enzyme, dNTP,  $MgCl_2$  and cDNA. As shown in Fig. 3, 0.2 unite enzyme and 0.001 mM dNTP (Table 1) were sufficient to amplify ApLV genome.

## DISCUSSION

To detect ApLV, fast and efficient optimization of RT-PCR was performed following the guidelines presented in Table 1. The combination of the optimized concentrations produced successful results in a stringent test using same RNA from three different tissue sources. No significant differences were observed either between optimized and standardized concentrations and/or values or between different plant tissues in detecting ApLV by RT-PCR.

At DNA template quantities between 0.8 and  $4/50 \mu\text{L}$  reaction volume, the amplification reaction showed no significant differences in all tried tissues; however, below  $0.8 \mu\text{L}$  the amount of some of the PCR products decreased. The success of PCR is partly based on its exponential amplification characteristics. Nevertheless, in practice, the achievable yields are limited (Czerny, 1996).

Primer concentrations may promote mispriming and accumulation of nonspecific product and may increase the probability of generating a template-independent artifact termed a primer-dimer. Nonspecific products and primer-dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTP and primers, resulting in a lower yield of the desired product (Innis and Gelfand, 1990). In general, the primers have to compete with the accumulating product in finding their target sequence, which might become a limiting factor for the reaction at later cycles. In practical terms, like cDNA concentration as the number of primer pairs was reduced the detection of the viral agent was inhibited. Thus, for a standard application a primer concentration between 20 and 100 pmol  $\mu\text{L}^{-1}$  is recommended for the detection of ApLV.

Both the specificity and the fidelity of PCR are increased by using lower dNTP concentrations. (Innis and Gelfand, 1990). Usta *et al.* (2005) found that low dNTP concentration resulted in a dramatic reduction in PCR yields.

In the absence of adequate free magnesium ion ( $\text{Mg}^{++}$ ), *Taq* DNA polymerase is inactive. Therefore, magnesium concentration is a crucial factor affecting the performance of PCR system (Eckert and Kunkel, 1990). For most applications, enzyme excess does not significantly increase the PCR yield. However, increased amounts of enzyme and excessively long extension times may increase the frequency of artifacts. These degradation artifacts are generated by the endogenous 5'-3' exonuclease activity associated with *Taq* DNA polymerase, which results in smearing of the PCR products in agarose gels (Sardelli, 1993). All concentrations tried in this experiment allowed PCR amplification of visibly higher amounts of products.

Increasing the concentration of PCR components such as dNTPs, primer,  $\text{MgCl}_2$ , cDNA and enzyme concentrations may increase the likelihood of mis-priming with subsequent production of spurious nonspecific amplification products (Chamberlain *et al.*, 1989). Alteration of these factors in uniplex PCR may result in improvement in the sensitivity or specificity of the test.

In general, these investigations confirmed that when using optimized PCR parameters, the two-step PCR system is capable of detecting the ApLV with high fidelity, that can reproducibly be used for three different plant tissues and that the sensitivity is significantly increased compared to the standard PCR concentrations or values.

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