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Simultaneous detection of *Apple Chlorotic Leaf Spot Virus* and *Apple mosaic virus* in crab apples and apple rootstocks by duplex RT-PCR

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ABSTRACT

Apple Chlorotic Leaf Spot Virus (ACLSV; family *Betaflexiviridae*, genus *Trichovirus*) and *Apple mosaic virus* (ApMV; family Bromoviridae, genus *Ilarvirus*) are economically important viruses of apple (*Malus* × *domestica* Borkh.). A duplex reverse transcription polymerase chain reaction (RT-PCR) assay was developed for simultaneous detection of both ACLSV and ApMV along with an internal control (NADH dehydrogenase subunit 5 gene). The internal control was used to minimize the risk of getting false negative results. Specific primers were designed against coat protein gene of ApMV while for ACLSV & internal control previously reported primer sets were used. Firstly the uniplex RT-PCR assay was standardized for the detection of each virus independently. Then duplex RT-PCR protocol for simultaneous detection of ACLSV and ApMV was standardized by using primer pairs ACLSV-CP1F & ACLSV-CP1R and ApMV-CP1F & ApMV-CP5R, respectively, along with primer set for internal control. Robustness of the technique was further validated wherein; duplex RT-PCR was carried out to detect both viruses in crab apples, rootstocks and different popular cultivars of apple. Results reveal that the duplex RT-PCR assay has detection sensitivity as that of uniplex RT-PCR assay for respective viruses. This optimized duplex RT-PCR provides a simple, rapid, sensitive and convenient way for simultaneous detection of ACLSV & ApMV by reducing the time and cost of the supplies.

1. Introduction

The apple (*Malus* × *domestica*) belongs to the family Rosaceae which is one of the most widely cultivated temperate fruit tree. About 69 million tons of apples were grown worldwide in 2010 and China produced almost half of this total whereas, India stands in 5th position with an area of 0.29 mHa, production of 2.89 mt and productivity of 10 t/Ha (Anonymous, 2012). Wild species of genus *Malus* that are relatives of the cultivated apple and that produce small sour fruit are called as crab apples. These crab apples may be used in breeding program, as pollinizers in apple orchards, ornamental trees and most commonly as rootstocks for domestic apples. Crab apple *Malus baccata* collected from Shillong is an ideal example of wild species being used as a rootstock.

The relatively low productivity of apple in India is due to several biotic and abiotic factors. Biotic factors comprise of various fungal, bacterial and viral diseases. Many viruses have been reported in apple (Nemeth, 1986) viz. *Apple mosaic virus* (ApMV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) that cause significant yield losses (Mink, 1989). ACLSV is a type member of the genus *Trichovirus* (Martelli et al., 1994) of *Betaflexiviridae* family (Adams et al., 2004), whereas ApMV belongs to the genus *Ilarvirus* and family Bromoviridae. ACLSV was first reported in *Malus* spp. from the US by Mink and Shay in 1959 (Burnt et al., 1996). It is one important latent virus whose infection rates ranges up to 80–100% in many commercial apple cultivars with yield losses to the tune of 30–40% (Nemchinov et al., 1995; Wu et al., 1998; Cembali et al., 2003). In Himachal Pradesh (India) ACLSV appeared as a major virus on apple with disease incidence ranging from 85% to 90% (Rana et al., 2010).

ApMV is one of the oldest known viral diseases of plants (Posnette and Cropley, 1952). This virus was first reported in *Malus domestica* from the USA by Bradford and Joley (1933). In India, mosaic symptoms were first noticed in Uttarakhand during 1957 (Bhargava and Bist, 1957). It infects all kinds of apple in commerce and appears sporadically but frequently. The virus spreads by means of vegetative propulage. The typical symptom of infection is color change of the leaf. An apple tree infected with the ApMV will display symptoms of pale to bright cream spots on the leaves. It causes reduction in shoot growth, fruit set, fruit weight, yield per

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tree and ascorbic acid content of the fruit (Singh et al., 1979) which results in yield reduction of up to 60% (Menzel et al., 2002). Similarly yield loss of more than 46% in the cultivar Golden Delicious was reported due to ApMV infection (Cembali et al., 2003).

Apparently healthy looking and high yielding plants used as a source of bud stick may carry latent virus infection. Similarly virus infected rootstock/crab apple multiplied clonally is also potential source of virus transmission. Hence detection of the virus in elite mother plants, rootstocks and in crab apples used as a rootstock is a matter of great importance. Although, a natural spread under field condition has been observed but the natural mode of spread is unknown. Thus a program of disseminating virus-free material will help in controlling virus infection. So, reliable detection of viruses is an important aspect in generation of virus-free planting material (Nakahara et al., 2011).

Biological indexing and ELISA are routinely used for detection of important viruses of apple. Both methods have their limitations, the major limitations of biological indexing are its application length (3 years), its cost in terms of glasshouse space and labor intensity and sometimes symptoms are difficult to interpret. At the same time ELISA often fails because of low virus titers or the inhibitory effects of plant polysaccharides or phenolic compounds. In addition, no internal controls are available to prevent false negative results in ELISA.

Therefore for routine diagnosis, reliable, fast, inexpensive and robust procedures are essential, therefore, PCR technique would provide a possible alternative. Use of duplex RT-PCR assay will further reduce the costs, time and quantity of sample required. Hence an experiment was design to standardize duplex RT-PCR for simultaneous detection of ACLSV and ApMV in a given sample.

2. Materials and methods

2.1. Samples for ELISA and RT-PCR

Leaf samples from rootstocks, crab apples and popular cultivars were collected from Indian Agricultural Research Institute Regional Station (IARI-RS), Shimla (HP), India and were stored at 4 °C until further processing. Leaf samples from virus infected plants maintained at IARI-RS, Shimla were used as a positive control in the present study. Lab-based techniques were carried out in the Division of Plant Protection, Central Potato Research Institute (CPRI), Shimla, HP (India) and the Division of Mycology and Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, HP (India).

2.2. ELISA

Leaves collected from different apple plants (Table 2) were then serologically subjected to detection of viruses through double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). For ELISA tests, reagents, buffers, controls supplied by the BIOREBA AG (Switzerland) were used as per the instructions of manufacturer. ELISA results were interpreted by following Lemmetty (1988) and Dijkstra and Jager (1998), wherein samples were considered infected when their OD values at 405 nm (A_{405}) exceeded two times the mean values of respective healthy and negative control samples.

2.3. Isolation of total RNA and c-DNA synthesis

Total RNA was isolated from leaves of apple plants by using Spectrum™ Plant Total RNA kit (Sigma–Aldrich, USA) as per manufactures instructions. The isolated total RNA was quantified by Thermo Scientific Nanodrop 2000. The first strand of c-DNA was synthesized by Revert Aid™ c-DNA synthesis kit (Fermentas Life Sciences) using random hexamer. The reverse transcription (RT) mixture comprising of 4.0 μl of 5× buffer, 2.0 μl of 10 mM each dNTP mix, 1.0 μl of 20 U/μl RNase inhibitor, 1.0 μl of 0.2 μg/μl random primer, 6.0 μl of template RNA, 1.0 μl of 200 U/μl RT enzyme and 5.0 μl of RNase-free water to provide a final volume of 20 μl. All the reactions were set up in ice cold condition to avoid premature cDNA synthesis and minimize the risk of RNA degradation. The reaction mixture was mixed, briefly centrifuged and incubated at 25 °C for 5 min, 42 °C for 59 min, 75 °C for 10 min. Later cDNA was used for further PCR amplification and the remaining quantity was stored at −20 °C for further use.

2.4. Designing of virus specific primers for RT-PCR

Coat protein gene was targeted to design primers for ApMV. Sequence data of ApMV (CP region: 6.677 of Acc no: FN435317.1) was obtained from the Genebank of the National Center for Biotechnology Information (NCBI) and primers were designed by using software Primer 3. With respect to ACLSV primer set ACLSV-CP1F, ACLSV-CP1R reported by Watpade et al. (2012) was used. The primer names, oligonucleotide sequences, and expected size of amplified products are shown in Table 1.

2.5. Primers for internal control

Performance of RT-PCR with an internal control can minimize the risk of obtaining false negative results (Menzel et al., 2002; Thompson et al., 2003). Although several plant internal control primers have been published (Bariana et al., 1994; Nassuth et al., 2000) none of them can distinguish DNA from RNA so that the complete removal of DNA is required before performing RT-PCR (Nassuth et al., 2000; Menzel et al., 2002). To solve this problem, Menzel et al. (2002) designed the primers for amplifying apple mitochondrial NADH dehydrogenase subunit 5 (nad 5) gene. The same primer set, i.e., sense: 5′-gatgcttcttggggcttcttgtt-3′ and antisense: 5′-ctccagtcaccaacattggcataa-3′ giving 181 base pair amplification was used in the present study.

2.6. Optimization of uniplex RT-PCR

For ACLSV, PCR protocol standardized by Watpade et al. (2012) was used. To optimize PCR conditions for ApMV, PCR was carried out in thin walled 1.25 ml tubes in GeneAmp PCR 9700 system (Applied Biosystems, USA). The reaction mixture of 20 μl containing 0.8 units of Red Taq DNA polymerase (Genei, Banglore, India) 1.6 μl of 2 mM dNTP mix (Fermentas), 2.5 μl of 10× taq DNA Polymerase buffer (Genei, Banglore, India), 0.5 μl of each downstream and upstream primers, 2 μl of cDNA and volume was made up to 20 μl with DEPC treated water. Amplification was carried out by following PCR conditions with five primer pairs along with an internal control. Denaturation was performed at 94 °C/30 s annealing temperature of 59 °C/1 min followed by extension at 72 °C/1 min for 40 cycles along with a final elongation step at 72 °C for 10 min. After PCR about 10 μl of the reaction mixture from each tube was loaded onto 1% agarose gel alongside 1 kb DNA ladder as molecular weight marker. Electrophoresis was carried out at 80 V, the buffer used was 1× TAE at 8.0 pH. The DNA bands in the gel were visualized on a UV-transilluminator and primer pair showing expected size was selected.

2.7. Optimization of annealing temperature

After selecting a primer set, same PCR mix and PCR conditions were used on varying annealing temperatures ranging from 57 °C to 63 °C (57 °C, 59 °C, 61 °C and 63 °C). Once the primer