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## Detection of *Apple Chlorotic Leaf Spot Virus* in Tissues of Pear Using *In Situ* RT-PCR and Primed *In Situ* Labeling

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### 1. Introduction

*Apple chlorotic leaf spot virus* (ACLSV) is the type member of the Trichovirus genus, the family *Flexiviridae* (Martelli et al., 1994; Adams et al., 2004) and is known to infect most pome and stone fruit tree species, including apple, peach, pear, plum, almond, cherry and apricot (Lister, 1970; Németh, 1986). ACLSV has a worldwide distribution and induces a large variety of symptoms in sensitive fruit trees (Németh, 1986; Dunez & Delbos, 1988; Desvignes & Boyé, 1989). However, In Japan, this virus is one of the causative agents of topworking disease and induces lethal decline in apple trees grown on Maruba kaido (*Malus prunifolia* var. *ringo*) rootstocks (Yanase, 1974). Other severe symptoms of stone fruit trees in Europe caused by ACLSV including bark split and pseudopox in plum, bark split in cherry, pseudopox and graft incompatibility in apricot and ring pattern mosaic in pear (Dunez et al., 1972; Desvignes & Boyé, 1989; Cieślińska et al., 1995; Jelkmann & Kunze, 1995). ACLSV has very flexuous filamentous particles, approximately 640 to 760 nm in length and consisting of a single-stranded positive-sense RNA with Mr of  $2.48 \times 10^6$  and multiple copies of a 22 kDa coat protein (CP) (Yoshikawa & Takahashi, 1988).

*In situ* detection techniques allow specific nucleic acid sequences to be exposed in morphologically preserved tissue sections. In combination with immunocytochemistry, *in situ* detection can relate microscopic topological information to gene activity at the transcript or protein levels in specific tissues. In certain cases, they also can provide increased specificity and more rapid analyses. *In situ* reverse transcription polymerase chain reaction (RT-PCR) is a molecular biological-cytological method. *In situ* RT-PCR combined the sensitiveness of PCR amplification with spatial localization of products to monitor the appearance of specific transcripts in the tissue sections. Therefore, *in situ* RT-PCR defined a powerful tool for the low abundance transcript detection (Pesquet et al., 2004). Hasse et al. (1990) first reported the *in situ* PCR technology, which combined the strong points of PCR and *in situ* hybridization. It was widely used for all kinds of disease and genetic studies in human and animal (Gressens & Martin, 1994; Staskus et al., 1991; Nuovo et al., 1991; Bagasra et al., 1992; Cohen, 1996; Chen & Fuggle, 1993; Höfler et al., 1995). The first application of *in situ* RT-PCR for the plant tissue was reported by Woo et al. (1995). Most recently, this

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method had not been used to a large extent in plants (Greer et al., 1991; Johansen, 1997; Matsuda et al., 1997).

The primed *in situ* labeling (PRINS) procedure is a fast and efficient alternative to conventional fluorescence *in situ* hybridization for nucleic acid detection. According to the PRINS method, laboratory-synthesized oligonucleotide probes are used instead of cloned DNA for the *in situ* localization of individual genes. The PRINS primers are annealed to complementary target sequences on tissues and are extended in the presence of labeled nucleotides (Koch et al., 1995) utilizing *Taq* DNA polymerase. Since its introduction, the PRINS protocol has been continuously optimized, and numerous applications have been developed (Thomas et al., 2001; Yan et al., 2001; Xu et al., 2002; Tharapel & Wachtel, 2006a, 2006b; Wachtel & Tharapel, 2006; Kaczmarek et al., 2007). The technique has thus proved to be a useful tool for *in situ* screening, and has become a simple and efficient complement to conventional and molecular cytogenetic methods.

In this paper, we optimized the *in situ* RT-PCR and PRINS method for increased sensitivity to localize the virus in plant tissues with ACLSV. Based on this research, through observing distribution of amplified cDNA in tissues, we can analysis the virus infection. In this way, it can provide a new approach to detection virus in fruit trees, as well as investigate the formation, distribution and transformation of virus and produce innocuity fruit trees.

## 2. Materials

### 2.1 Virus sources

Leaves were collected from Korla pear in Shayidong commercial orchard of Korla, Xinjiang, China. Virus-free healthy leaves were used as negative controls.

### 2.2 Reagents and enzymes

*Taq* DNA Polymerase, dNTPs, dATP, dGTP, dCTP, dTTP, PMD19-T were all purchased from TakaRa (China); M-MLV Reverse Transcriptase, T4 DNA ligase were from Fermentas (USA); TIANprep Mini Plasmid Kit and TIANgel Midi purification Kit were from TIANGEN (China); SuperScript II RNase H-Reverse Transcriptase were from Invitrogen (EU); Proteinase K were from Merk (Germany); Digoxigenin-11- dUTP, alkaline phosphatase labeled anti-digoxin, anti-digoxin- fluorescence, Ribonuclease inhibitor, DNaseI were purchased from ROCH (USA); Nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro- 3-indolyphosphate (BCIP) were purchased from Shanghai Sangon (China); others were all analysis purity made in China. *E. coli* DH5 $\alpha$  as preserved strains were stored at Biotechnology Laboratory of Horticultural Department, Agriculture College, Shihezi University, China.

### 2.3 Primer design

The sequences were amplified by *in situ* RT-PCR reaction with specific primers, which were designed according to the cDNA sequence of ACLSV (Sato et al., 1993). Primer sequences are as follows: forward primer (P3) 5'-GGCAACCCTGGAACAGA-3' and the reverse primer (P4) 5'-CAGACCCTTATTGAAG TCGAA-3'.

The sequences were amplified by PRINS reaction with specific primers, which were designed according to the cDNA sequence of ACLSV from GenBank D14996 (Table 1). A

Blast search of the primer sequences showed that they were specific for their intended targets.

Primer	Primer Sequence (5'-3')	Annealing Temp (°C)
acls Pa 1	CTTTACGAGCCCATTCTTGCC	61.5
acls Ps 1	GAACATAGCGATACAGGGGACC	60.3
acls Pa 2	TGCCTCACACACTTGGCGGAG	60.6
acls Ps 2	CGATACAGGGGACCTCGGAAC	61.5
acls Pa 3	GCCTTTACGAGCCCATTCTTG	59.5
acls Ps 3	AGGGGACCTCGGAACAAACAG	60.5
acls Pa 4	GTACAAAAGAGGTTTGTGAAG	54.2
acls Ps 4	GTGCTGGTGGAGGTGAAATC	57.4
acls Pa 5	CAATCTGAAGGAGGTAGTCGGT	56.4
acls Ps 5	TTCAGGCGTAGTAGAAAAGAGG	57.7

Table 1. Oligonucleotide primers used to PRINS

### 3. Methods

#### 3.1 Total RNA extraction and RT-PCR

Total RNAs were extracted from phloem infected by ACLSV. The 200 mg fresh Pear phloem tissue were grinded in liquide nitrogen for a fine powder and transferred to a 1.5 mL eppendorf tube which has added 800  $\mu$ L extraction buffer (50 mmol  $\cdot$  L<sup>-1</sup> Tris-Cl pH 8.0, 140 mmol  $\cdot$  L<sup>-1</sup> NaCl, 10 mmol  $\cdot$  L<sup>-1</sup> EDTA, 4% SDS, 3% PVP, 15% ethanol, 5%  $\beta$ -mercaptoethanol), well mixed by inversion of the tube. Added 500  $\mu$ L Tris-saturated phenol (pH 8.0): chloroform: isoamyl alcohol (25: 24: 1) to the tube, sepaeated by centrifugation at 12 000 rpm for 15 min at 4°C. Transferred the supernatant by hand-suction to a fresh tube and mixed with an equal volume of Tris-saturated phenol (pH 8.0): chloroform: isoamyl alcohol (25: 24: 1), followed by centrifugation at 12 000 rpm at 4 °C for 15 min. The supernatant was transferred to a fresh tube and mixed with an equal volume of chloroform: isoamyl alcohol (24: 1) and then centrifugation at 12 000 rpm at 4°C for 10 min. Transferred the supernatant to a fresh tube and added 2.0 volumes of LiCl. Precipitated at -20°C for 2-3 h. RNA was separated by centrifugation at 12 000 rpm for 15 min at 4°C. Removed the supernatant by hand-suction, washed the pellet two times by 70% ethanol, air-dry at room temperature. Suspended the pellet in 20-30  $\mu$ L of TE solution or DEPC-treated sterile water and analysed it immediately by electrophoresis or stored at - 20°C.

The reverse transcription mixture contained 1.0  $\mu$ L specific reverse primer and 5.0  $\mu$ L of total RNA and 9.5  $\mu$ L of ddH<sub>2</sub>O. The mixture was kept at 70°C for 5 min, and then immediately transferred to ice for 5 min. Then 2.5  $\mu$ L of dNTPs (10 mM each), 5.0  $\mu$ L of 5 $\times$ M-MLV buffer, 1.0  $\mu$ L of RNasin ribonuclease inhibitor (40 U  $\mu$ L<sup>-1</sup>), 1.0  $\mu$ L of M-MLV reverse transcriptase (200 U  $\mu$ L<sup>-1</sup>) and made the total volume of 25.0  $\mu$ L. The mixture was incubated at 42°C for 1 h.

PCR reaction volumes were 20.0  $\mu\text{L}$ , and contained 2.0  $\mu\text{L}$  of 10 $\times$ PCR buffer, 0.5  $\mu\text{L}$  of dNTPs (each 10 mM), 2.0  $\mu\text{L}$  of primers, 2.0  $\mu\text{L}$  of cDNA, 0.2  $\mu\text{L}$  (5U  $\mu\text{L}^{-1}$ ) *Taq* DNA polymerase and 13.3  $\mu\text{L}$  of ddH<sub>2</sub>O. PCR was carried out with an initial denaturation of 4 min at 94°C, followed by 35 cycles of 30s, 94°C; 30s, 55°C; 1 min, 72°C; and then by a final elongation step of 7 min at 72°C.

### 3.2 Cloning and sequencing

The amplified PCR products were gel purified and extracted using TIANGel Midi Purification Kit (TIANGEN, China). The purified DNA fragments were ligated into the PMD19-T vector (TaKaRa Biotechnology, China) following the manufacturer's instruction, and used to transform *E. coli* DH5 $\alpha$ . The positive clones were confirmed by PCR and restriction enzyme digestion before sequencing. Two clones from independent PCR reactions were sequenced from both directions.

### 3.3 Tissue embedding and preparation of slide

1. Slide disposal: After rinsed, ultrasonic cleaned and high temperature baked, the slide must be pre-prepared with poly-L-lysine for 5 min, and then incubated it at 26°C overnight, sealed and stored at room temperature for use within 10 d.
2. Tissues fixation: Leaves were cut into small pieces (3 $\times$ 2 mm) and rinsed the tissues in 4% paraformaldehyde immediately for 1h at room temperature with gentle shaking.
3. Dehydration: Washed the tissues in PBS buffer two times (5 min each), immersed the tissues in series of concentration of ethanol (50%, 70%, 85%, 95% and 100%) for 1h, respectively, at room temperature.
4. Transparencies: Put the tissues into pure alcohol: xylene (1: 1) and pure xylene for 1 h, respectively, at room temperature.
5. Low-temperature wax infiltration: Put the tissues into the container which contained transparency and paraffin, covered the container with lid, and incubated at 38°C overnight.
6. High-temperature wax infiltration: Removed the lid, and put the container into incubator at 58°C, and then changed the pure paraffin three times for 2 h each.
7. Paraffin-embedding: Pour melted paraffin wax to pre-folded carton for embedding.
8. Sectioning: Tissue sections (2-16  $\mu\text{m}$ ) were obtained by a conventional rotary microtome. If very thin sections were required, a retracting rotary microtome should be used to avoid the compression of the tissue block by the up-stroke of the knife and sections should be mounted onto poly-L-lysine-coated pre-prepared slides.
9. Stretched section: Wax sections needed to be stretched before adhesion to the glass slide. Sections were lifted onto a layer of de-gassed water on a slide held on a warmed flat plate (45°C). Once the sections was stretched, drained away the excess water and left the slide into incubator at 40°C, overnight, the section has dried onto the slide, stored at -20°C.

### 3.4 Pretreatment of slides

1. De-waxed: Removed the slides from the refrigerator, put the slide into the oven incubated for 1-3 h, at 60°C in order to melt paraffin. Rinsed the slide in xylene for 5 min

- and transferred to ethanol for 5 min, repeated more times until the paraffin was completely removed, then left the slide at room temperature for air-dry.
2. Proteinase K treatment: Added  $1 \mu\text{g mL}^{-1}$  Proteinase K digested 10-45 min at  $37^\circ\text{C}$ , stopped reaction by washings for 5 min in PBS buffer and transferred to DEPC-treated sterile water for 5 min at room temperature, then air-dry.
  3. DNaseI treatment: For each slide,  $4.0 \mu\text{L}$   $10\times$ DNase I buffer,  $4.0 \mu\text{L}$  DNase I ( $10 \text{ U } \mu\text{L}^{-1}$ ),  $1.0 \mu\text{L}$  Ribonuclease inhibitor ( $40 \text{ U } \mu\text{L}^{-1}$ ) and DEPC water added to  $20.0 \mu\text{L}$  in a  $0.5 \text{ mL}$  microtube. Applied the reaction solution onto the slide and put it into humidified chamber and incubated at  $37^\circ\text{C}$  overnight.
  4. Wash the slide two times in DEPC-treated sterile water for 5 min each and in alcohol for 5 min at room temperature.

### 3.5 *In situ* reverse transcription reaction

For each slide,  $4.0 \mu\text{L}$   $5\times$ Frist-Strand Buffer ( $\text{MgCl}_2^+$   $15 \text{ mM}$ ),  $2.0 \mu\text{L}$  dNTPs ( $10 \text{ mM}$  each),  $1.0 \mu\text{L}$  RNasin ( $40 \text{ U } \mu\text{L}^{-1}$ ),  $1.0 \mu\text{L}$  Antisense primer ( $20 \mu\text{M}$ ),  $2.0 \mu\text{L}$  DDT ( $0.1 \text{ M}$ ),  $1.0 \mu\text{L}$  SuperScript II RT ( $200 \text{ U } \mu\text{L}^{-1}$ ), and DEPC water added to  $20.0 \mu\text{L}$  in a  $0.5 \text{ mL}$  microtube. Applied the reaction solution onto the slide and put it into a humidified chamber and incubated at  $42^\circ\text{C}$  for 1 h, then inactivated at  $92^\circ\text{C}$  for 1 min. Washed the slide two times for 5 min each in distilled water at room temperature.

### 3.6 *In situ* RT-PCR detection

#### 3.6.1 *In situ* RT-PCR reaction

The reaction was consisted of  $2.5 \mu\text{L}$   $10 \times$  PCR buffer ( $\text{Mg}^{2+}$  free),  $0.5 \mu\text{L}$  dNTP ( $10 \text{ mmol } \mu\text{L}^{-1}$ ),  $1.0 \mu\text{L}$  each primer ( $20 \text{ pmol } \mu\text{L}^{-1}$ ),  $2.5 \mu\text{L}$  Dig-11-dUTP ( $1 \text{ nmol } \mu\text{L}^{-1}$ ),  $1.0 \mu\text{L}$  *Taq* DNA polymerase ( $2.5 \text{ U } \mu\text{L}^{-1}$ ) and distilled water to  $25.0 \mu\text{L}$ . Mounted the slide with genic frame, added the reaction solution, and covered the slide with a cover slip, then put the slide on the flate bloke of the thermocycler. Cycling parameters consisted of  $94^\circ\text{C}$  for 3 min,  $94^\circ\text{C}$  for 2 min and 35 cycles of a two-step PCR with an annealing temperature of  $56^\circ\text{C}$  for 1 min. Removed the cover slip and inactivated at  $94^\circ\text{C}$  for 2 min. Washed the slid two times for 10 min each in washing buffer with gentle shaking. Several slides were used as negative controls for each *in situ* RT-PCR experiment. One slide was healthy plant, the other slides were amplified without primers, *Taq* DNA polymerase, or RT step.

#### 3.6.2 Immunoenzymatic detection

1. Mounted the slide with  $100 \mu\text{L}$  blocking buffer ( $100 \text{ mmol } \cdot\text{L}^{-1}$  Tris-HCl, pH 7.5,  $150 \text{ mmol } \cdot\text{L}^{-1}$  NaCl, and 3% BSA). Incubated the slide in a humidified chamber at  $37^\circ\text{C}$  for 30min. Drained the blocking buffer from the slide.
2. Added anti-Dig-alkaline phosphatase (1: 100 in blocking buffer), and incubated the slide in a humidified chamber for 30 min at room temperature.
3. Stopped the reaction by rinsing the slide with washing buffer ( $100 \text{ mmol } \cdot\text{L}^{-1}$  Tris-HCl, pH 7.5,  $150 \text{ mmol } \cdot\text{L}^{-1}$  NaCl) two times for 10 min each at room temperature with gentle shaking.
4. Developed the color reaction by adding  $100 \mu\text{L}$  of NBT/BCIP solution to the slide and incubated the slide in a humidified chamber for 60 min in the dark at room temperature. Then rinsed the slide with water to stop the reaction.

5. Rinsed the slide in series of concentration of ethanol, 50%, 70%, 85%, 95%, and 100% for 2 min, respectively, at room temperature for dehydration.
6. Put the slide into pure xylene for 3 min for transparent.
7. Covered the section with the cover slip using mounting solution, air-dry. Then the sections were ready for data recording, which could view under bright field microscopy through stained with Alcian Blue.

### 3.7 PRINS detection

#### 3.7.1 PRINS reaction

1. Immersed slides in 0.02 N HCl for 20 min.
2. Denature the samples by immersing them in 70% formamide/2×SSC, at 72°C for 2 min.
3. Dehydrate the slides in a series (70%, 90%, and 100%) of ice-cold ethanol washes (4°C) before allowing them to air-dry.
4. Prepare reaction mixture in a final volume of 25.0  $\mu\text{L}$  consisted of specific primers (20  $\mu\text{M}$ ) 10.0  $\mu\text{L}$ , 0.1% BSA 2.5  $\mu\text{L}$ , 0.2 mM dNTPs 2.5  $\mu\text{L}$  (each), 0.02 mM dTTP 1.0  $\mu\text{L}$ , 0.02 mM Dig-11-dUTP 3.0  $\mu\text{L}$ , *Taq* buffer 2.5  $\mu\text{L}$ , *Taq* DNA polymerase (2.5 U  $\mu\text{L}^{-1}$ ) 1.0  $\mu\text{L}$  and distilled water to 25.0  $\mu\text{L}$ . Kept the mix on ice during preparation and until application to the slide.
5. Reaction mixture incubated at annealing temperature and incubated the denatured the slide for 7 min at annealing temperature. Applied the reaction mixture and covered the working area of the slide completely with a 22  $\times$  22 cover slip on the denatured the slide, and then transferred to the heating block of the thermal.
6. Set up the PRINS program and start the reaction. The program was carried out on a programmable thermal cycler equipped with a flat plate for slides. The program consisted of one cycle of 9 min at annealing temperature with an additional 30 min at 72°C for extension.
7. After extension, the slide was removed from cycler, the cover slip was removed, and the slide washed in NE solution (500 mM NaCl, 50 mM EDTA, pH 8.0) at 72°C for 5 min, and transferred the slide to 4×SSC/0.2% Tween-20 at 50°C for 5 min to stop the reaction.

#### 3.7.2 Visualization of PRINS products

1. For each slide, added 10  $\mu\text{g mL}^{-1}$  avidin-Rhodamine and 20  $\mu\text{g mL}^{-1}$  anti-digoxigenin-FITC.
2. Placed slides in a humidified chamber for 30 min at room temperature, worked in the dark as much as possible to avoid fluorescence bleaching.
3. The slide was rinsed in preheated solutions (1×PBS/0.2% Tween-20, 37°C; 0.5×PBS/0.2% Tween-20, 37°C; 0.2×PBS/0.2% Tween-20, 37°C) for 5 min, respectively, air-dried.
4. Mounted the slide with 3  $\mu\text{g mL}^{-1}$  of DAPI/antifade solution under a 22×22 coverslip counterstained for 10 min, in dark.
5. Let the excess mounting medium dry. Approximately 1 h, permanently seal the slide with nail polish. Slide can be maintained at 4°C until scored.

#### 3.7.3 Signal detection and image analysis

Olympus BX51 fluorescence microscope system was adopted for this process. This system contained Olympus UPlanFI 100×/1.30 Oil  $\infty$ /0.17 C1field lens, pass band filter with

DAPI/FITC/Rhodamine, AxioCam Camera module and Video Test-FISH 4.0 image analysis system.

## 4. Results

### 4.1 Detection ACLSV by RT-PCR

Total RNA were extract from the phloem of pear which were infected with ACLSV, first strand cDNA synthesis was obtained by reverse transcription using specific primer and 358 bp fragment was amplified by P3/P4 primers as shown in Figure. 1. The purified DNA fragments were ligated into the PMD19-T vector and transformed into *E. coli* DH5 $\alpha$ . The positive clones were confirmed by PCR and restriction enzyme digestion before sequencing.

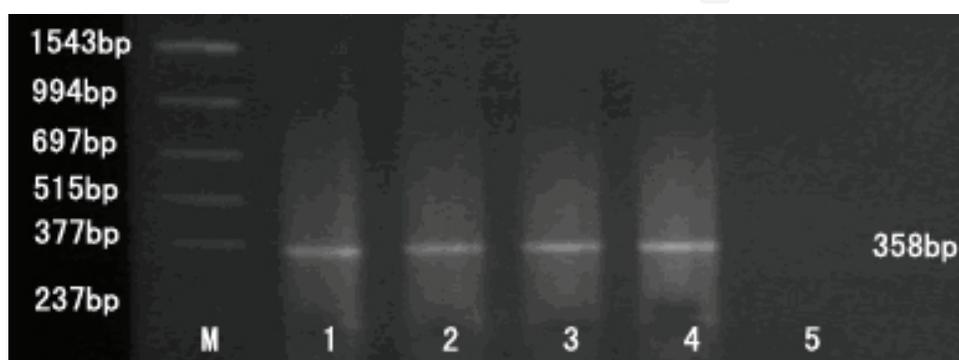


Fig. 1. The productions of RT-PCR of ACLSV  
M: Marker; 1-4: productions; 5: negative control

### 4.2 Detection the reliability of alkaline phosphatase chromogenic system

The slide were digested by  $1\mu\text{g mL}^{-1}$  Proteinase K for 20 min at  $37^{\circ}\text{C}$ , and incubated at  $37^{\circ}\text{C}$  overnight with DNase I. Washed the slide two times for 10 min each in PBS buffer. Mounted the slide with blocking buffer and incubated at  $37^{\circ}\text{C}$  for 30min. Added anti-Dig-alkaline phosphatase (1:100 in blocking buffer) and incubated the slide in a moist chamber for 60 min at room temperature, then washed the slide two times for 10 min each in PBS buffer at room temperature with gentle shaking. Added NBT/BCIP solution to the slide and incubated the slide in a humidified chamber for 60 min in the dark at room temperature. The result showed that sections were not stained.

### 4.3 The effect of treatment with proteinase K

After treated with Proteinase K treatment for 10 min or 15 min, the organization performed a piece of blue, which indicated that Proteinase K digested inadequately. Morphology was fuzzy when digested for 30 min or 40 min, illustrating excessive digestion. Proteinase K treatment 20 min was more moderate.

### 4.4 The effect of RT-component concentration

The results showed there was no signal when RNasin was less than  $0.2\text{ U}\cdot\mu\text{L}^{-1}$ , and it was enhanced with the increased RNasin. The concentration of dNTPs was above  $0.4\text{ mmol}\cdot\text{L}^{-1}$ , the signal was appeared; the concentration of SuperScript II ranged from  $0.1\text{U}\cdot\mu\text{L}^{-1}$  to  $1.3$

$U \cdot \mu L^{-1}$  and the signal was enhanced with the increase concentration of SuperScript II; the concentration of primers above  $0.9 \mu mol \cdot L^{-1}$  were effective, less than  $0.8 \mu mol \cdot L^{-1}$  could not synthesized sufficient quantities of cDNA and above  $1.2 \mu mol \cdot L^{-1}$  could produce non-specific product.

#### 4.5 The effect of other factors

The result showed that positive signals were appeared on the slide only when the annealing temperature at  $56^{\circ}C$ , which indicated that the suitable temperature was  $56^{\circ}C$ . Amplification with 10-20 cycles, the signals were not appeared, 25 cycles appeared weaker blue signal, 30-35 cycles showed stronger signals, which demonstrated that fewer cycles led to lower

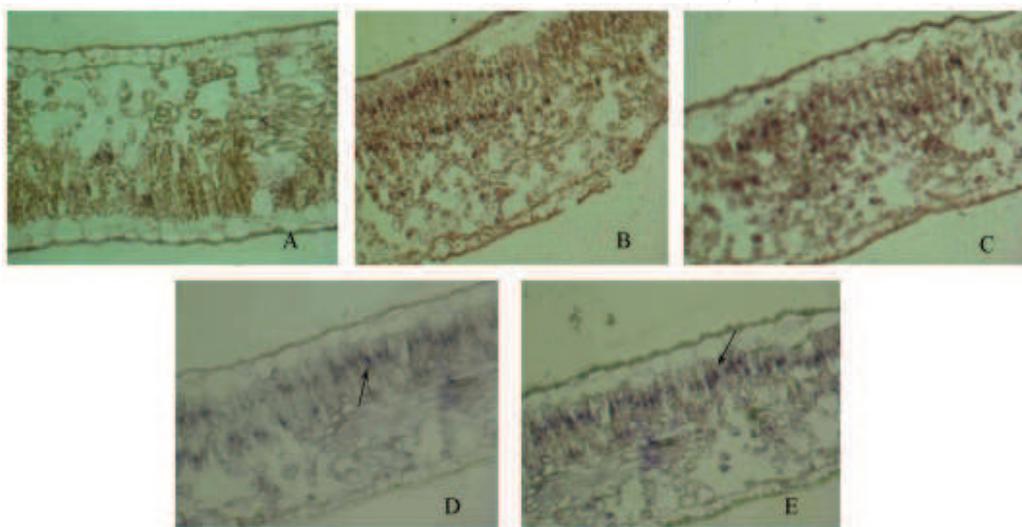


Fig. 2. The effect of cycle number on *In situ* RT-PCR  
A: 10 cycles; B: 15 cycles; C: 20 cycles; D: 25 cycles; E: 30 cycles

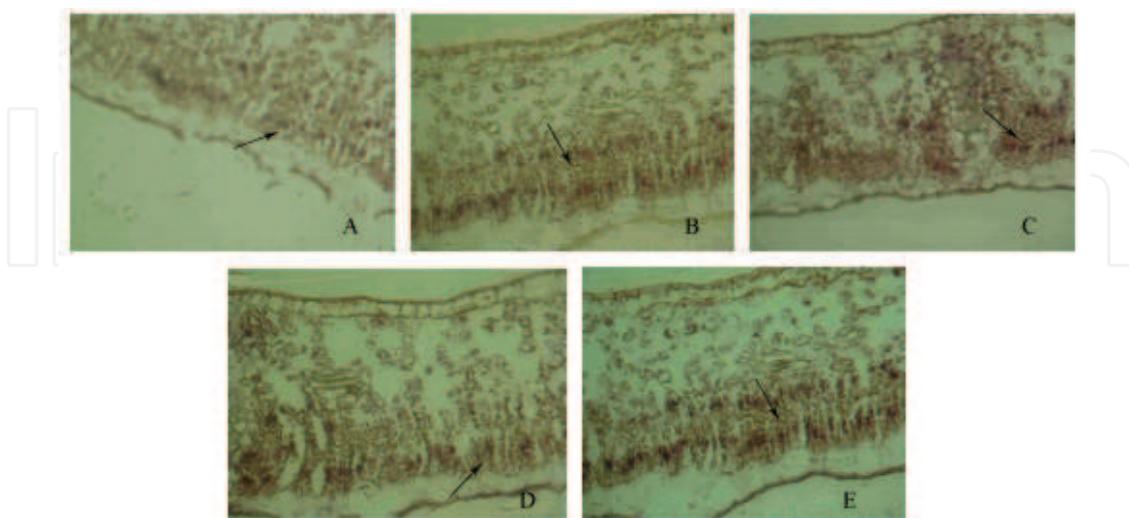


Fig. 3. The effect of the different *Taq* DNA polymerase concentration on the detection of *In situ* RT-PCR  
A:  $2 U \cdot 100 \mu L^{-1}$ ; B:  $4 U \cdot 100 \mu L^{-1}$ ; C:  $6 U \cdot 100 \mu L^{-1}$ ; D:  $8 U \cdot 100 \mu L^{-1}$ ; E:  $10 U \cdot 100 \mu L^{-1}$

synthesis (Figure. 2). The concentration of *Taq* DNA polymerase with  $2 \text{ U} \cdot 100\mu\text{L}^{-1}$ - $10 \text{ U} \cdot 100\mu\text{L}^{-1}$  could satisfy amplification and showed stronger signals, which indicated that the suitable concentration of *Taq* DNA polymerase was  $2 \text{ U} \cdot 100\mu\text{L}^{-1}$  (Figure. 3).

#### 4.6 PRINS-Rhodamine staining

Applied PRINS-Rhodamine staining detected ACLSV showed that the infected leaves of pear tissues were presented red fluorescence positive signals (Fig. 4, A~D, arrows showing the locations), which were consistent with the results of *In situ* RT-PCR detection (Niu et al., 2007). Healthy leaves and infected leaves without SuperScript II RT, fluorescent antibody and *Taq* DNA polymerase, did not present red fluorescence signals (Fig. 5, E~H).

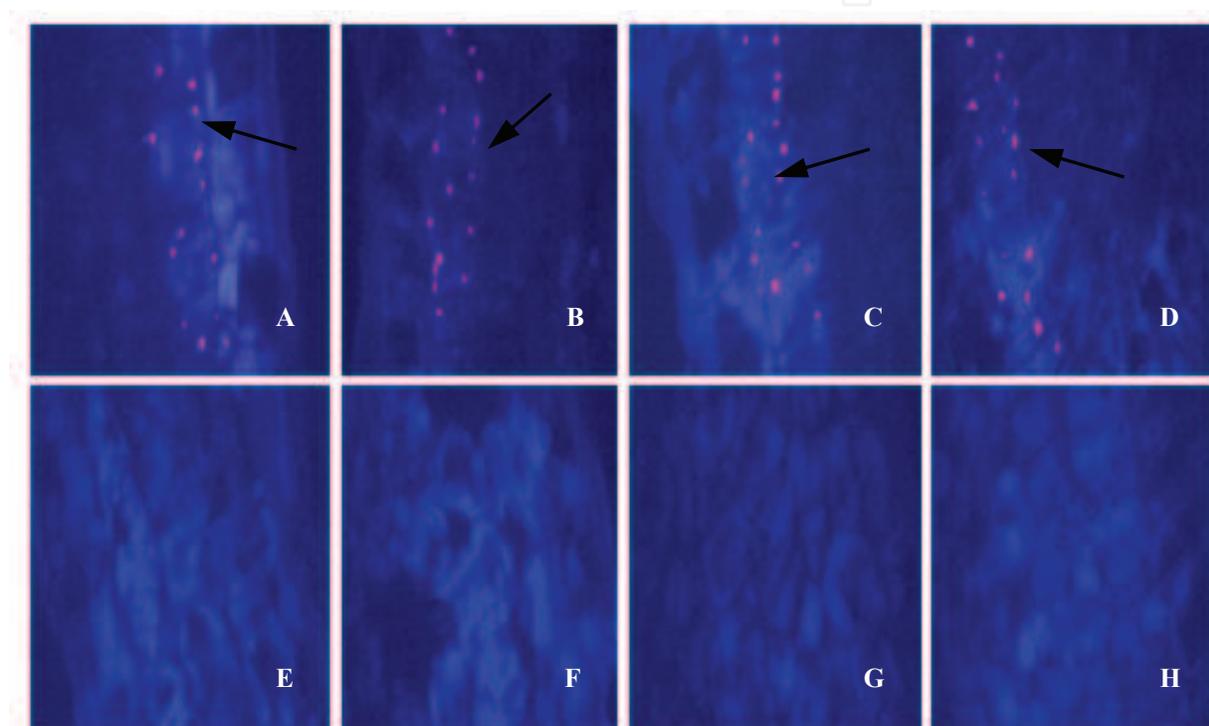


Fig. 4. PRINS-Rhodamine staining results of ACLSV in pear tissues  
A-D: Labeled results of virus infected pear leaves from the same positions of different trees;  
E: Labeled results of healthy pear leaf (control); F-H: PRINS-Rhodamine staining results of  
ACLSV in pear tissues (control: Left out of SuperScript II RT, fluorescence antibody, *Taq*  
enzyme).

#### 4.7 PRINS-FITC staining

FITC fluorochrome was more sensitive to the temperature and pH, and the efficiency was lower than Rhodamine staining, and the results showed inconspicuous signals. Applied PRINS-FITC staining detected ACLSV showed that the infected leaves of pear tissues were presented green fluorescence positive signals (Fig. 5, A~D, arrows showing the locations), which were consistent with the results of *In situ* RT-PCR detection (Niu et al., 2007). Healthy leaves and infected leaves without SuperScript II RT, fluorescent antibody and *Taq* DNA polymerase, did not present red fluorescence signals (Fig. 2, E~H).

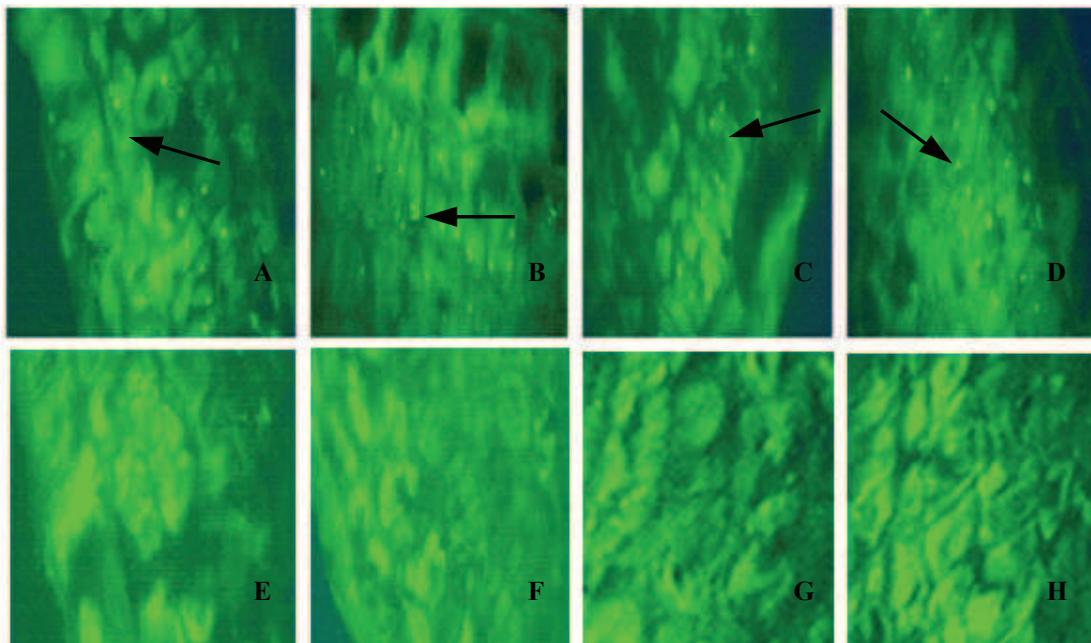


Fig. 5. PRINS-FITC staining results of ACLSV in pear tissues  
 A-D: Labeled results of virus infected pear leaves from the same positions of different trees;  
 E: Labeled results of healthy pear leaf (control); F-H: PRINS- Rhodamine staining results of ACLSV in pear tissues (control: Left out of SuperScript II RT, fluorescence antibody, *Taq* enzyme).

## 5. Discussion

The study is based on virus RNA as a template to reverse transcription cDNA and *in situ* amplification. Before amplification, the slides treatment with DNA exonuclease without RNA enzyme overnight digest the original genomic DNA in tissues which can eliminating DNA fragment decorated by polymerase which could form false-positive amplification (Long et al., 1993). In our studies, the known virus-free material of pear tree used as the negative control did not appear specificity of fluorescence signals. Negative control without SuperScript II RT, fluorescence antibody, *Taq* enzyme showed the same result of virus-free material. Signals did not display without RT steps indicated that the products were amplified by cDNA, which excluded the possible of experimental reagents cross produced fluorescent complex and attached to the tissue surface induced fluorescence signals. In our studies, ACLSV of leaf sections of Korla Pear were detected by *in situ* RT-PCR and PRINS, the results showed that the positive materials were found obviously alcian blue and fluorescence signals in mesophyll cells, while the negative control tissue did not appear. It was indicated that ACLSV mainly distributed in the palisade tissue of the mesophyll cells, and the same results as *in situ* RT-PCR detection (Niu et al., 2007). In addition, the results showed that the thickness of section had a great influence on detection. Thin slices can easy to cause the tissues were not complete, and the cell of thick slices were multiple and overlapping, which unfavorable for observing, and seriously affect the detection results. So, in order to obtain desire results of detection, the 4-6  $\mu\text{m}$  of sections were used.

Because of the *in situ* amplified cDNA in tissues, we must consider the number of primers to use. A single primer would not allow a strong enough signal for fluorescent detection.

However, too many primers would likely lead to primer-dimers or non-specific hybridization. In PRINS reaction system, primer extensions strictly followed the principle of complementary base pairing, and ensure the specificity labeling. Synthesis of labeled DNA will remain in the amplified position and not diffusion. In this study, we used five specific primers for PRINS, and achieved clearly fluorescence signals.

Terkelsen et al., (1993) using repeated primed *in situ* labeling (repeated-PRINS). This change of strategy results in a localized accumulation of sequence-specific labeled DNA, resulting in up to a 15-fold amplification of the signal as compared to the standard PRINS method. Ni et al., (1998) results showed that the repeated-PRINS technology could to enhance the signal; however, repeated heat denaturation and extension process for long time which induced the cell loss normal forms. In our study, we pretreatment species with appropriate concentration of protease K, and the optimal time of proteinase K digestion was necessary. The tissues slices were treated with proteinase K for 10, 20, 30, and 45 min. The best results were achieved after 20 min of the proteinase K digestion. The morphology of the tissue was well retained, and interpretation of results was unambiguous. The signal was recognized as fluorescence-signals the site of the label. The 10 min durations turned out to be too short and led to lack of signal. The extension of the reaction time up to 45 min produced morphological distortions to the point that interpretation of results became impossible. In addition, our research showed that increasing the ratio of dTTP and labeled-dUTP could improve the signal intensity. In general, the ratio of dTTP and labeled-dUTP was 1: 1 could generate enough strong signals. We increased the dTTP and labeled-dUTP concentration ratio to 1: 3 generated strong signals.

In this study, two fluorescence labeling were used, FITC and Rhodamine, respectively. Fluorescent-FITC was used *in situ* labeling showed sensitive on PH and easy to decay. In the conditions of susceptible pH or strong UV irradiation, the fluorescence excitation rapid decay. In addition, increase the times of washing, the tissues were more easily damaged and higher backgrounds were obtained. Therefore, on the basis of complete elution, appropriate to reduce washing processing steps were necessary.

Primed *in situ* labeling (PRINS) of nucleic acids was developed as an alternative to traditionally used fluorescence *in situ* hybridization (FISH). PRINS is based on sequence-specific annealing of unlabelled oligonucleotide primer under stringent conditions to the DNA of denaturated. Compared to FISH, PRINS is faster and does not require preparation of labeled probes, the process costs much less in terms of reagents (Velagelati et al., 1998; Tharapel & Kadandale, 2002; Pellestor et al., 2002), and hybridization signal is stronger, more specific and easy to control. In addition, we believe that this modified PRINS technique can have very meaningful applications in molecular cytogenetics. It can be used for the visualization and mapping of genetic loci on chromosomes, and for detection of the presence or absence of small DNA segments involved in genetic diseases. PRINS will have a more extensive application prospects in plant virus detection.

## 6. Conclusions

ACLSV of leave sections of Korla Pear were detected by *in situ* RT-PCR and PRINS, and the positive materials were found obviously alcian blue and fluorescence signals in mesophyll cells. The results showed that *in situ* RT-PCR and PRINS, which had two staining methods

of PRINS-FITC and PRINS-Rhodamine, could get good detection results in which the parts have viruses showed alcian blue, green and red fluorescence light, respectively. Therefore, primed *in situ* labeling technique can be perfectly used for virus *in situ* detection of fruit trees, and it is also a rapid, simple and reliable *in situ* detection method.

## 7. Acknowledgements

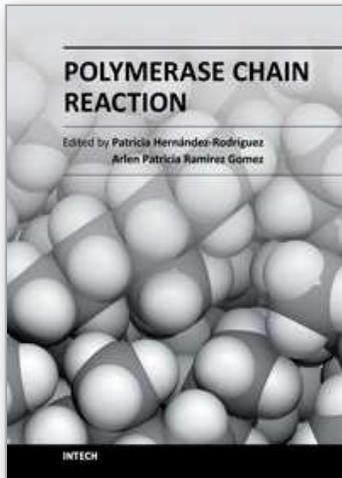
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