INCIDENCE OF SWEET CHERRY VIRUSES IN SHANDONG PROVINCE, CHINA AND A CASE STUDY ON MULTIPLE INFECTION WITH FIVE VIRUSES

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SUMMARY

Leaves were collected from a total of 62 trees displaying virus-like disease symptoms such as rugose mosaic in leaf, irregular shaped leaf blades or small leaf blades in a survey of sweet cherry (*Prunus avium* L. cv Red Lamp) in Shandong Province, the largest cherry production area in China. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Little cherry virus-2* (LChV-2), *Cherry virus A* (CVA) and *Cherry green ring mottle virus* (CGRMV) were present and that most of the samples (70%) were infected by at least two viruses. A case study in this survey of two selected trees revealed that a mixed infection with these five viruses simultaneously caused two types of field symptoms: late-maturity in fruits or fruit blast. Real-time quantitative RT-PCR analysis revealed that the relative proportion of the virus titers was distinct in the two sampled trees. The PNRSV titer was significantly higher than the four other viruses in the trees that exhibited fruit blast. Phylogenetic analysis of the PNRSV coat protein (CP) gene showed that the isolates HSY-4-1 and X-3, obtained from the two sampled trees separately, were classified into different subgroups of Group I isolates, which is characterized by a six-nucleotide insertion. A non-synonymous substitution was identified in the six-nucleotide insertion in the PNRSV CP gene in one of the two isolates. This is the first detailed study of the incidence of multiple sweet cherry viruses in China.

Key words: *Prunus necrotic ringspot virus*, *Prune dwarf virus*, *Little cherry* virus-2, *Cherry virus A*, *Cherry green ring mottle virus*, sweet cherry, RT-PCR, phylogenetic analysis of Ilarviruses.

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INTRODUCTION

Disease caused by viruses and virus-like agents is a serious problem in sweet cherry (*Prunus avium* L.) orchards in recent China, resulting in damaging effects on vegetative growth and fruit yield. However, a detailed report of virus infection in sweet cherry orchards, including the scale and the influence of infection on sweet cherry, and specifically the presence of mixed virus infections, is not available at present. Shandong Province is the most important production area of sweet cherry in China. By the end of 2010, over 50,000 ha were planted to sweet cherry with a total production of 220,000 metric tons, representing 45.6% of the total cultivation area and 62.9% of the total production in China. It is also the largest supplier for sweet cherry nursery stock, exporting to other production areas, including Shanxi, Sichuan and Gansu etc. In this article, we report the first study of the occurrence of viruses in sweet cherry in the Shandong Province using reverse-transcription polymerase chain reaction (RT-PCR), the purpose of which was to evaluate the incidence of mixed virus infections, which can have biological, epidemiological and economic implications, in sweet cherry in China.

To date, nine virus species have been identified in stone fruit in China. They include *Prunus necrotic ring spot virus* (PNRSV), *Prune dwarf virus* (PDV), *Apple mosaic virus* (ApMV), *Apple chlorotic leafspot virus* (ACLSV) (Zhou *et al*., 1996), *Cherry green ring mottle virus* (CGRMV) (Zhou *et al*., 2011), *Cucumber mosaic virus* (CMV) (Tan *et al*., 2010), *Cherry virus A* (CVA) (Rao *et al*., 2009), *Little cherry virus-2* (LChV-2) (Rao *et al*., 2011) and *Cherry necrotic rusty mottle virus* (CNRMV) (Zhou *et al*., 2013). Among them, the *Ilarvirus* species PNRSV, PDV and ApMV have been reported as the most serious threats and are a continued concern for sweet cherry commercial production (Pallás *et al*., 2012). Due to their similar routes of transmission, these three viruses usually infect the host simultaneously (Guo *et al*., 1995; Sánchez-Navarro and Pallás, 1997; Hammond and Crosslin, 1998; Fonseca *et al*., 2005). As co-infection with more than one virus species usually leads to synergism and an increase in symptom severity in host plants, we initiated a case study on the effect of simultaneous

natural infection of sweet cherry trees with multiple viruses to determine if symptomatology could be correlated to relative virus concentrations. We compared field symptoms, identified the virus species present in the infection, and performed a relative quantification of each virus. In addition, we performed a phylogenetic analysis of the CP gene in two isolates of PNRSV obtained from individual trees exhibiting different symptoms to establish if there was a correlation between the phylogenetic grouping and symptomatology.

MATERIALS AND METHODS

Plant materials and primers for RT-PCR detection. To survey viruses in sweet cherry trees in Shandong Province, 62 leaf samples were collected from nine orchards during 2011 and 2013, including 12 samples from Taian (two locations), 17 samples from Feicheng county (three locations), 21 samples from Xintai county (three locations) and 12 samples from Yantai (one location). All of the samples were obtained from the cultivar 'Red Lamp', which was considered to be the most important commercial cultivar with the largest cultivation area in China. The samples were selected from 62 trees exhibiting rugose mosaic in leaves, irregularly shaped leaf blades or small leaf blades, all of which were considered as the symptoms of virus-like diseases. The samples were frozen in liquid nitrogen and stored at –70ºC for further use. Leaf samples of the sweet cherry rootstock Gisela 6, obtained from shoot-tip culture by cryotherapy, were used as a negative control to show the specificity of the amplification. Sterile ddH_2O was used as another negative control to show no contamination in reagents. Amplified fragments of the PNRSV, PDV, LChV-2, CVA and CGRMV coat protein (CP) genes, which were cloned into pMD18-T vectors in our previous work, were used as the positive controls.

In the survey, ten virus species were selected as the targets for RT-PCR detection. Primers for the detection of PNRSV, PDV and CGRMV were referred to in previous reports (Zong *et al.* 2011; Isogai *et al.* 2004). Primers for CVA, LChV-2, *Cherry rasp leaf virus* (CRLV), *Cherry mottle leaf virus* (CMLV), *Little cherry virus-1* (LChV-1) and *Cherry necrotic rusty mottle virus* (CNRMV) were designed based on viral sequence data in Genbank. The sequences of the primers are shown in Table 1.

RNA extraction and RT-PCR detection. Total RNA for RT-PCR detection was extracted from the plant samples using a CTAB-based method according to the protocols of Gasic with some modifications in sample amounts (Gasic *et al.*, 2004). Briefly, frozen leaf tissue (100 mg ~ 300 mg) was ground in liquid nitrogen using a mortar and pestle and transferred into a microcentrifuge tube containing 1 mL of CTAB buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/l spermidine and 2% β-mercaptoethanol), vortexed and incubated at 65ºC for 10 min and centrifuged at 15,000*g* for 1 min. The samples were further processed according to the protocols of Gasic *et al.* (2004). The resulting total RNA was dissolved in 50 µl nuclease-free water. The quality and concentration of RNA was estimated by agarose gel electrophoresis and by evaluation in a Nanophotometer P330 (Implen, Germany). The samples collected from different locations were extracted separately to avoid cross contamination between samples.

For cDNA synthesis, reverse transcription was carried out using the random hexamer primer according to instructions of Fermentas RevertAidTM First Strand cDNA Synthesis kit (Fermentas, Thermo Scientific, Beijing, China).

For PCR detection, the cDNA (1µl) was used for amplification performed in an Eppendorf Mastercycler Gradient 5331 (Hamburg, Germany) in a total volume of 25 µl using the virus-specific primers listed in Table 1 and Taq PCR Mastermix (TIANGEN, China) at 95ºC for 5 min for denaturation and 35 cycles of amplification (95ºC for 50 s, 55ºC for 50 s and 72ºC for 50 s), with a final step of 72ºC for 10 min. The PCR products (10 µl) were analyzed in 1.5% agarose gel electrophoresis.

Field symptoms recording in the case study on multiple infections with five viruses. In order to evaluate the severity of the multiple infections with viruses, the field symptoms of the trees found to be simultaneously infected with five viruses in this survey were recorded in 2011 and 2013. Blooming dates, morphology of the flowers, symptoms in leaf blades and the development of the fruits were observed.

Quantitative analysis of the viruses in the case study. No. 35 and No. 40 sample trees, identified by RT-PCR detection as containing a mixed infection of the same five viruses, were selected for further analysis, mainly because they were located in different orchards, were infected with the same virus species, and exhibited altered fruit development. Flowers were taken separately for the analysis.

Total RNA was extracted as described above and treated with RNase-free DNase (Promega, USA) to eliminate genomic DNA contamination. cDNA was synthesized according to the same protocols above. Fluorescent real-time quantitative PCR was performed on an ABI 7500 Real-time Detection System (Applied Biosystems, USA) to determine the relative expression of the five viruses. CP gene-specific primer pairs PNRSV-Fr/Rr, PDV-Fr/Rr, LCV2-Fr/Rr, CVA-Fr/Rr and CGRMV-Fr/Rr (Table 1) were used to amplify the products of 195 bp, 112 bp, 107 bp, 188 bp and 88 bp, respectively. The sweet cherry housekeeping gene *β-actin*, which was amplified using primer pair ACTIN-Fr/Rr, was selected as the internal control to calibrate the cDNA template of the corresponding samples. PCR amplifications were carried out

in triplicate in a total volume of 20 µl containing 10 µl of 2×SYBR qPCR Mix (MBI Fermentas), 0.4 µl of each primers (5 µmol/l), 1 µl of a 1:10 diluted cDNA and 8.2 µl ddH_2O. The PCR program was 95°C for 10 min, followed by 50 cycles of 30 s at 95°C, 30 s at 60°C. A melting curve analysis was added according to the instrument default program, and consisted of 95°C for 15 s, followed by 60°C for 15 s and finally 95°C for 15 s, to confirm the specificity of amplification. The expression level of each gene was analyzed by the comparative CT ($2^{-\Delta\Delta CT}$) method.

Sequence alignment and phylogenetic analysis of the PNRSV isolates in the case study. The PNRSV CP gene was amplified from the No. 35 (type B; HSY4-1) and No. 40 (type A; X3) samples and cloned into pMD18-T plasmid vector (TaKaRa, Japan) for sequencing. Sequence alignment and phylogenetic analysis were made using MEGA 6.0 software program by means of the neighbor-joining method. Corresponding sequences were submitted to GenBank.

RESULTS

Molecular detection of cherry viruses from sweet cherry trees. Electrophoretic analysis of the RT-PCR products revealed the presence of PNRSV, PDV, CVA, LChV-2 and CGRMV, with the PCR products of the expected sizes obtained (data not shown). In contrast, no fragment with expected size was amplified using primer pairs for ACLSV, CRLV, CMLV, LChV-1 or CNRMV (data not shown). These results demonstrate that the samples collected in this survey are infected with at least five viruses. As summarized in Table 2, PNRSV

Table 1. Oligonucleotide primers for detection of various viruses by RT-PCR and real-time quantitative RT-PCR (qRT-PCR).

Primers	Sequence (5' to 3')	Accession Number	Product Size
RT-PCR			
PNRSV-S	TGGTCCCACTCAGGGCTCAACAAAG	NC_004364	455bp
PNRSV-A	ACGCGCAAAAGTGTCGAAATCTAAA		
PDV-S	CGAAGTCTATTTCCGAGTGG	NC_008038	304bp
PDV-A	CCACTGGCTTGTTTCGCTGT		
CVA-S	ATGCTTCGCAGGTGACGATA	NC_003689	652bp
CVA-A	GCTTGTTTGTGGAGGGAGAC		
CGRMV-S	TAAACCCCTGCAATTCCACTC	NC_001946	192bp
CGRMV-A	CTCTAAGGAAACTGAAGGAAAA		
LChV2-S	TCCGAATAGTCAGTTCAAAG	NC_005065	337bp
LChV2-A	AATAGCCCTCATAAATCTCC		
LChV1-S	GACGAGCGTGAGATTGAGGA	NC_001836.1	556bp
LChV1-A	CTTTGTGCATTGGTGTAGCG		
ACLSV-S	GGTAATCCTGGAACAGA	AJ243438.1	358bp
ACLSV-A	AAGGCCCTTATTGAAGTCAAA		
CRLV-S	GTGGTCATCTTGCTGGTTAT	AY122330.2	775bp
CRLV-A	GGTATAGAAATGCGAACAGAG		
CMLV-S	GACTCTTCAGGGTTGGTTCG	NC_002500.1	425bp
CMLV-A	CTCAATGTGATTTCGCAAGG		
CNRMV-S	GGGCAGAGCCAAATGTAAT	NC_002468.1	243bp
CNRMV-A	CTAATGAGTCCAGCCACAGG		
qRT-PCR			
PNRSV-Fr	TGAAGGACCAACCGAGAG	NC_004364	195bp
PNRSV-Rr	CGACCAGCAAGACATCAG		
PDV-Fr	ACGTTCCGGTGTGGTTTTGTA	NC_008038	112bp
PDV-Rr	ATCGCATCCATAAGGGCAGTGT		
LChV2-Fr	TGATGCAGGTAGAGACGGTTTG	NC_005065	107bp
LChV2-Rr	GAGCATTGTGTATTGTTTTTGACG		
CVA-Fr	GCGGTGGAGGCATCAGAG	NC_003689	188bp
CVA-Rr	TCTATCACTGGTCTCGGGATTG		
CGRMV-Fr	GTGGGGTTGGACTTGAA	NC_001946	88bp
CGRMV-Rr	CACACTACTGGAGCATACTTT		
ACTIN-Fr	CCTGGACTATGAGCAAGAAC	FJ560908	142bp
ACTIN-Rr	AATGAGTGATGGTTGGAAGAG		

Table 2. Detection of PNRSV, PDV, CVA, LChV-2 and CGRMV in sweet cherry trees.

Virus	PNRSV	PDV	CVA	LChV-2	CGRMV
Infection (%)	29/62 (46.8)	37/62 (59.7)	42/62 (67.7)	37/62 (59.7)	43/62 (69.4)

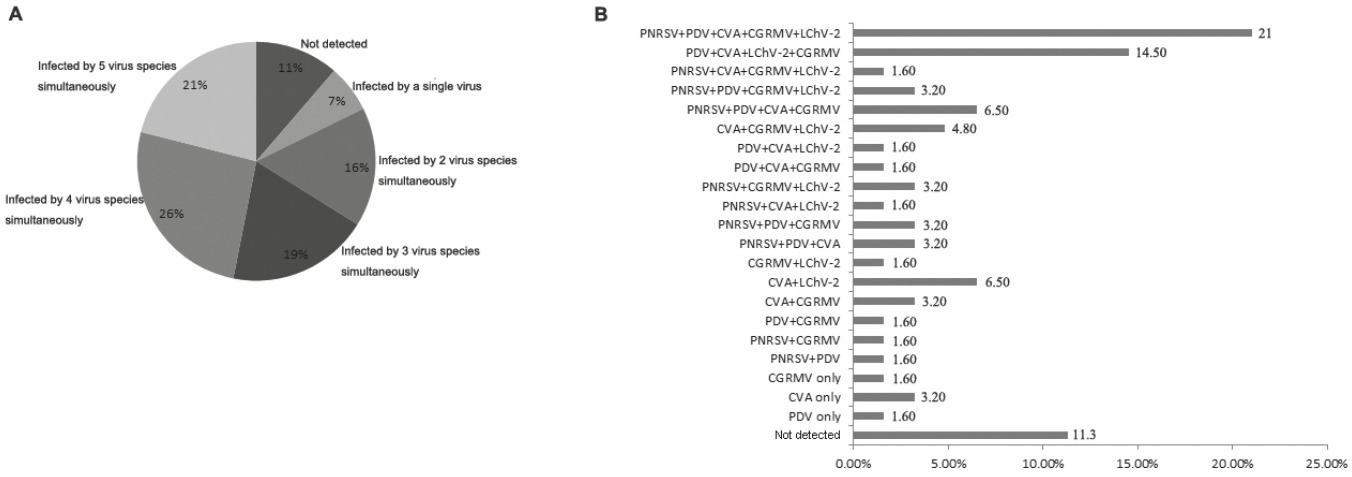

Fig. 1. Statistical data illustrating virus incidence in the 62 field samples in this study. (A) Frequency of infection with single, double, triple, quadruple or quintuple virus species in sweet cherry samples. (B) Bar graph illustrating the percent of samples infected with single or multiple viruses and the viruses present in mixed infections.

and PDV were detected in 29 of 62 samples (46.8%) and in 37 of 62 samples (59.7%), respectively. CVA and CGRMV were detected in a much higher proportion compared to the other three viruses identified in this survey. CVA was detected in 42 of 62 trees (67.7%) and CGRMV was detected in 43 of 62 samples (69.4%). Further analysis indicated that the viruses usually infected the host in a mixed infection. As shown in Fig. 1A, approximately 82.3% of the samples were infected by at least two virus species. Nearly half the samples (29 of 62 samples) were infected simultaneously by four or five virus species. The infected virus species of each sample were recorded and summarized in Fig. 2B. This analysis revealed that 21% of the samples were infected by the virus group 'PNRSV+PDV+CVA+CGRMV+LChV-2' and 14.5% were infected by the virus group 'PDV+CVA+CGRMV+LChV-2'. These two groups represented a much higher proportion of infection than those of other infected samples. It also appeared that some viruses frequently occurred in the presence of a particular virus in the mixed infection. For example, among the 29 PNRSV- positive samples, 24 were simultaneously infected with PDV, and 32 of the 37 LChV-2-positive samples in total were also infected with CVA (Fig. 1B).

Comparison of the field symptoms in the case study on multiplex infection with five viruses. The field symptoms of the trees sampled in this survey were recorded from 2011 to 2013. The trees naturally infected with five viruses simultaneously drew more attention primarily because although the trees were positive for the same collection of virus species, different symptoms were evident.

The symptoms were primarily represented by two types: those plants that produced fruit normally and those that did not bear fruits. As shown in Fig. 2A (type A) and Fig. 2B (type B), some symptoms were common among the two types. When compared to healthy trees (disease-free defined as not containing any of the viruses that were assayed for), the blooming date of the infected trees was almost five days later and the petals were smaller and wrinkled. The leaves appeared to be rugose with thin and irregular shapes. Chlorotic spots were unevenly distributed between the interveinal areas of the upper leaf-surface. However, fruit development showed different phenotypes**.** In Fig. 2B, during the early stage of fruit development, no evident distinction was observed. But two weeks after full blossom, the fruits in the infected trees did not fully develop and finally dropped. The infected trees seldom bore mature fruits. However, as shown in Fig. 2A, cherry fruits could fully develop to maturity, but the fruit suture was deeper and more obvious compared with the fruits of healthy trees and the shape of the fruit was asymmetrical along the suture. Due to the late flowering in the disease-infected trees, the fruit maturity was postponed for five to seven days correspondingly.

Quantitative analysis of the five viruses in the case study. In order to reveal the factors possibly causing different symptoms in cherry fruit development, the relative proportion of the virus titers was determined. Two sampled trees from different orchards, which were represented as type A and type B corresponding to the symptoms described in Fig. 2, were selected in the case study. As shown in Fig. 3, some similar characteristics in virus gene expression could be detected in both type A and type B sampled trees. The expression levels of *LChV2-CP* and *CGRMV-CP* were much lower than the other three virus genes in both trees. However, the relative proportion of each virus titer in flowers was different in the two sampled trees. The expression of *PDV-CP* was slightly higher than *PNRSV-CP* and *CVA-CP* in the type A sampled tree. On the other hand, *PNRSV-CP* was highly expressed and there was no significant difference between *CVA-CP* and *PDV-CP* in the type B sampled tree.

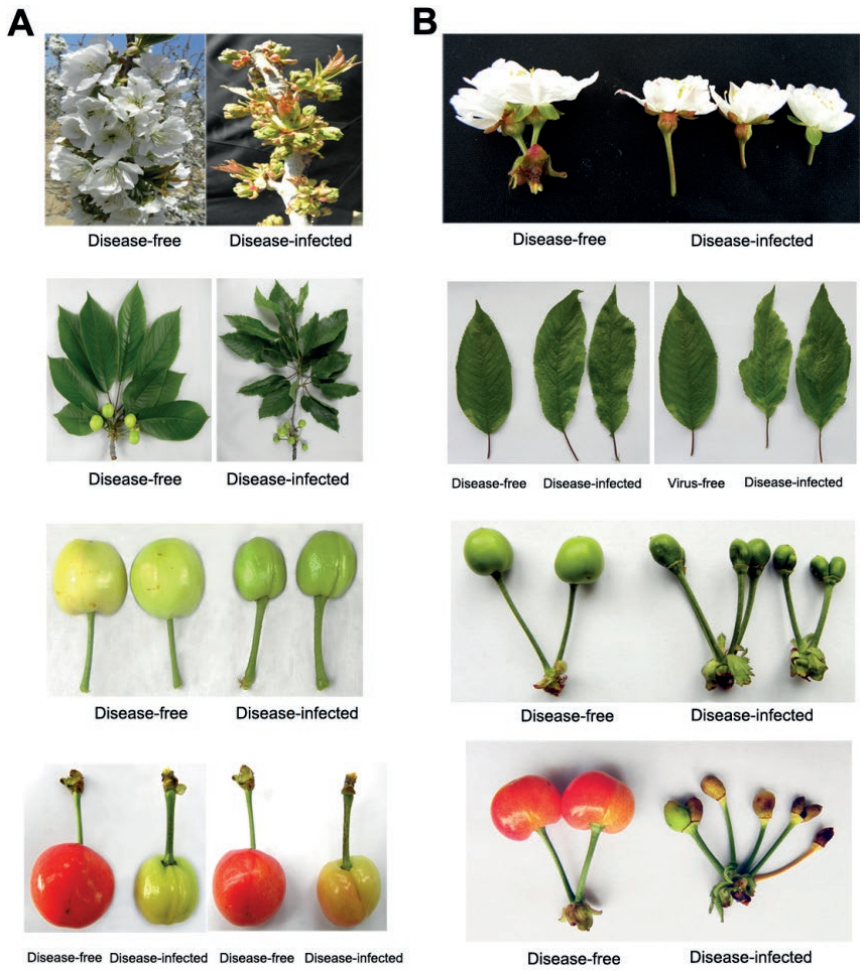

Fig. 2. Field symptoms observed in sweet cherry trees infected simultaneously with PNRSV, PDV, CVA, CGRMV and LChV-2. (A) Type I, late flowering time observed, however, cherry fruits could fully develop to maturity. The fruit suture was deeper and more obvious compared with the fruits in the healthy trees and the shape of the fruit was asymmetrical along the suture. (B) Type II, late flowering time observed and fruits blasting.

Sequence analysis of PNRSV coat protein genes in the case study. It was reported by Pallas *et al.* that *Ilarvirus* species are a continued concern for fruit trees and PNRSV may cause chlorotic or yellow line pattern mosaic in leaves and delay in fruit maturity (Pallás *et al.*, 2012). This description was similar to the symptoms observed in our study. Previous reports indicated that the phylogroups of PNRSV isolates tended to exhibit different symptoms in hosts, mild or severe (Aparicio *et al.* 1999; Aparicio and Pallás, 2002; Hammond, 2003). Thus, the phylogenetic relationships of PNRSV isolates from the two sampled trees were analyzed based on sequences of CP gene. As indicated in Fig. 4A, both the isolates HSY-4-1 (type B) and X-3 (type A) clustered into Group I (PV32), of which some isolates have been reported to be virulent. Further comparison of sequence polymorphisms within the CP genes was carried out, primarily because the two isolates were classified into different subgroups within Group I (Fig. 4A). As indicated in Fig. 4B, while HSY-4-1 and X-3 possessed the common sequence features of Group I, including a six-nucleotide insertion at position 1226 (resulting in

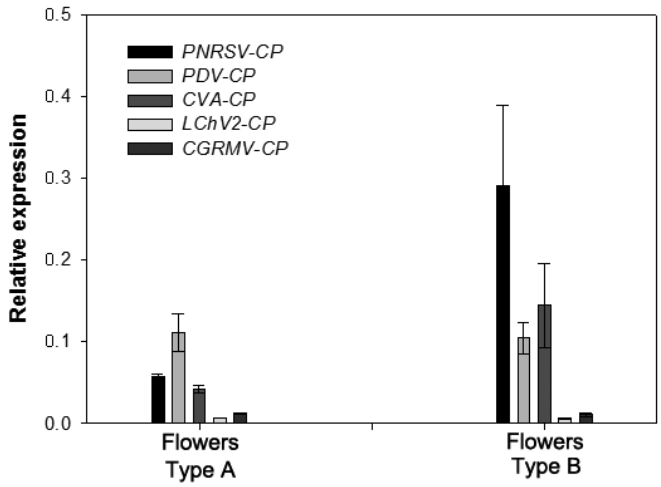

Fig. 3. Relative expression of the CP gene of five viruses in flowers collected from two sampled trees in the case study. It was analyzed by qRT-PCR using primers listed in Table 1. Expression (y-axis) was calculated relative to the expression of *β-actin* (at zero). Standard deviation is represented by vertical lines above the bars.

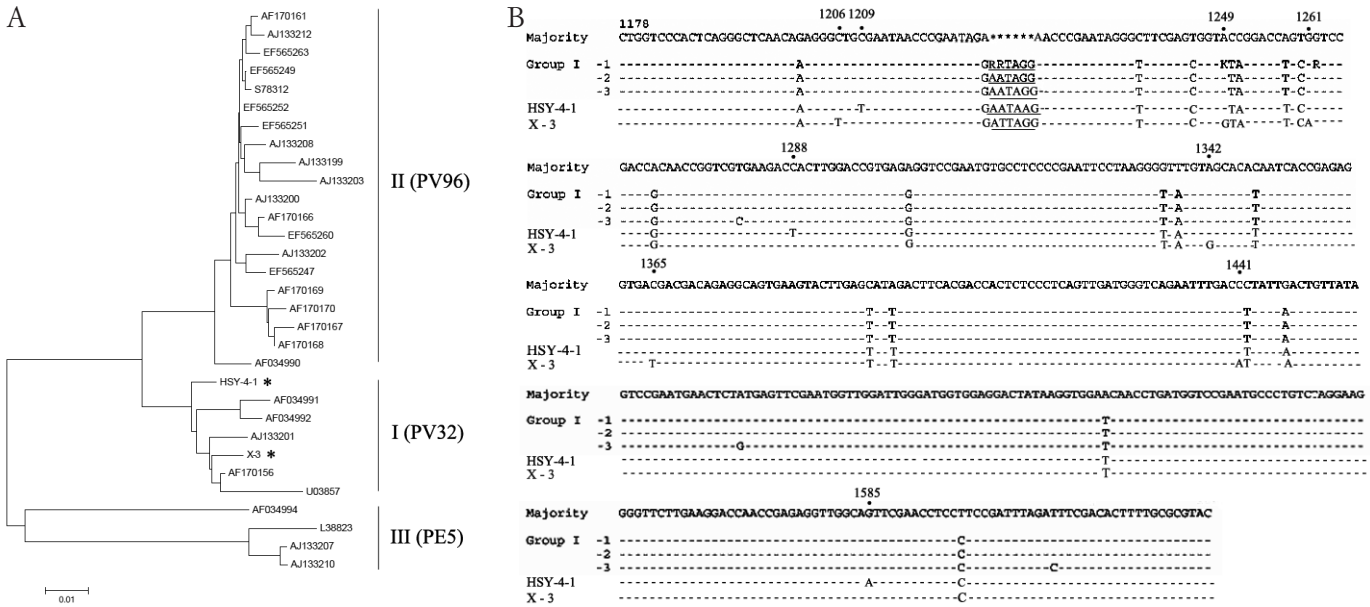

Fig. 4. Phylogenetic analysis and multiple alignment of the nucleotide sequences of the CP genes of PNRSV. (A) Phylogenetic tree of PNRSV CP gene sequences characterized in this study (*). Branch lengths are proportional to the number of inferred characters. Group designations on the right reflect major groupings of PNRSV isolates. (B) Nucleotide features of Group I isolates. The majority sequence was generalized from the available representatives of all three groups of PNRSV. Subgroups of Group I, designated as 1, 2 and 3, are presented as described in Hammond (2003). Asterisks represent nucleotides gaps of major groups. The six-nucleotide insertion in Group I is underlined. The special nucleotides sites in HSY-4-1 and X-3 are denoted by a dot (·) over the majority sequence.

a two amino acid insertion in the CP) of RNA3, there are also several point mutations. C→T transitions occurred at 8 different positions, nt. 1238, 1245, 1251, 1260, 1348, 1393, 1442 and 1597, G→A transitions occurred at 5 different positions, nt. 1201, 1225, 1270, 1303 and 1447, and A→T tranversions occurred at position 1258, 1338, 1396 and 1519. According to this analysis, the frequency of C→T transition was much higher than the other nucleotides transition or transversion types. Additional nucleotide polymorphisms that occur in subgroups within Group I were also present. For example, HSY-4-1 included the C→T transition at position 1209 as well as position 1288 and G→A transition at position 1585, which are characteristic of Group III (PE5), while X-3 did not possess these transitions contained the majority sequence of subgroup-1, -2 and -3 (Hammond, 2003). In addition, it is notable that the six-nucleotide insertion occurring in HSY-4-1 was 'AATAAG' and in X-3 it was 'ATTAGG', which caused a non-synonymous substitution of amino acid residues.

DISCUSSION

Several recent papers have reported the incidence of stone fruit viruses in China. PNRSV, PDV, ACLSV and ApMV were first detected by ELISA in China in 1996 (Zhou *et al*., 1996). During 2009 to 2011, a number of viruses such as CVA, CGRMV and LChV-2 were subsequently identified (Rao *et al*., 2009; Rao *et al*., 2011; Zhou *et al*., 2011). These papers described a first report of a single virus but did not include other extensive information. Shandong Province plays a key role in the production of sweet cherry in China. Surveys on the incidence of virus disease in this district could provide some guidance to the rest of the country for virus testing, not only because this province is the largest cultivation area of sweet cherry trees, but it also holds the dominant position in nursery stock exporting to the rest of the country.

In this study, we tested cherry leaf samples by RT-PCR for the presence of ten virus species and found that the samples were positive for up to five of these viruses. The detection rates for CVA and CGRMV, at 67.7% and 69.4% respectively, were much higher than those for PNRSV, PDV, and LChV-2. A high infection rate with CVA was previously observed in Japan, where 92% of the trees tested were infected with this virus (Isogai *et al*., 2004). However, there was no evidence that this virus was associated with any known disease. It was hypothesized that CVA infection may not be significant individually, but may enhance the severity of symptoms when combined with other viruses (James and Jeckmann, 1998). CVA was originally found in a cherry tree affected by little cherry disease (Jelkmann, 1995), however LChV-2 was later shown to be the main pathogen of little cherry disease (Rott and Jelkmann, 2001). In our study, there appeared to be an association between infection with CVA and LChV-2. For example, there were 37 LChV-2-positive samples in total and 32 of them were also infected with CVA. PNRSV and PDV, thought to be the most serious pathogens in sweet cherry commercial production, also appeared to infect

the hosts in a similar manner. In our study, among the 29 PNRSV positive samples, 24 of them were simultaneously infected with PDV. Such mixed infections have also been reported in other regions, such as Japan (Isogai *et al*., 2004) and California (Sabanadzovic *et al*., 2005).

In this study, in order to make a preliminary inquiry about the effects of mixed virus infection on cherry production, we showed a case study on the sampled trees that were infected by five viruses simultaneously based on RT-PCR detection. The field symptoms were mainly divided into two types: those that produced fruit (type A) and those did not bear fruits (type B). Late flowering time was commonly observed in both types (Fig. 2). It can be inferred that multiplex virus infection may affect the development of flowers and fruits in cherry trees. Since the sampled trees were infected by the same virus combinations, what factors lead to the different phenotypes in fruit development?

As an initial inquiry into the potential causes of the different phenotypes observed, we first compared the relative proportions of each virus titer in the flowers taken from the sampled trees. LChV-2 and CGRMV accumulated less than the other three viruses in both samples. The relative proportion of PNRSV titers was different, making up the highest proportion in type B-sampled trees. Although a correlation between high PNRSV titers and the phenotypes from a case study cannot be proven, it proposes an interesting question that may be further analyzed by a larger sample statistics and by experimental infection of trees by single and multiple viruses.

Phylogenetic relationships of PNRSV isolates were reported to be a potential factor that leads to different symptoms in hosts (Aparicio *et al*. 1999; Aparicio and Pállas, 2002; Hammond, 2003). The CP sequences of the HSY-4-1 and X-3 variants of PNRSV reported here revealed that the PNRSV variants grouped with cherry isolates predominantly considered to produce severe symptoms (Group I) and containing a six-nucleotide insertion in the coding region (Hammond, 2003; Hammond and Crosslin, 1998). Based on the limited data on pathogenicity of PNRSV isolates considered in the earlier study (Hammond, 2003) a strong correlation between symptom severity and group association based on the CP sequence could not be drawn, although there was a general trend for severe isolates to cluster into Group I. A nonsynonymous substitution of amino acid residues caused by substitution in six-nucleotide insertion was observed in sequences alignment (Fig. 4B). However, it is unclear if there is a biological significance of the six-nucleotide insertion in the CP gene. A recent study suggests that PNRSV RNA1 and RNA2, encoding proteins required for virus replication, co-determine viral pathogenicity, and that differences in pathogenicity are not determined by genomic RNA3 alone which encodes the movement protein and CP (Cui *et al*., 2013).

Finally, other potential causes might also be taken into consideration to explain the difference in fruit development between the type A and type B phenotypes, for example, the composition of the pollination cultivars surrounding the sampled trees may influence fruit set. In addition, although the same virus combinations were identified from the sampled trees by RT-PCR, it was not determined if other pathogens were present in the sampled trees. Deep-sequence analysis for pathogen identification may be a more efficient means to identify all of the pathogens present in the infected trees. In summary, our study revealed some details about the incidence of virus disease in sweet cherry within the largest stone fruit production area in China. To our knowledge, this is the first detailed investigation of sweet cherry virus infection in China.

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