To detect five plant viruses (Beet black scorch virus, Beet necrotic yellow vein virus, Eggplant mottled dwarf virus, Pelargonium zonate spot virus, and Rice yellow mottle virus) for quarantine purposes, we designed 15 RT-PCR primer sets. Primer design was based on the nucleotide sequence of the coat protein gene, which is highly conserved within species. All but one primer set successfully amplified the targets, and gradient PCRs indicated that the optimal temperature for the 14 useful primer sets was 51.9°C. Some primer sets worked well regardless of annealing temperature while others required a very specific annealing temperature. A primer specificity test using plant total RNAs and cDNAs of other plant virus-infected samples demonstrated that the designed primer sets were highly specific and generated reproducible results. The newly developed RT-PCR primer sets would be useful for quarantine inspections aimed at preventing the entry of exotic plant viruses into Korea.

**Keywords**: Beet black scorch virus, Beet necrotic yellow vein virus, Eggplant mottled dwarf virus, Pelargonium zonate spot virus, Rice yellow mottle virus

With the worldwide increase in trade among nations during last two decades, the number of exotic plants, including crops, fruits, and vegetables, that have been imported into Korea, has dramatically increased. Preventing the entry or spread of exotic pathogens that frequently accompany this plant material into Korea and into other countries relies in part on quarantine inspections. Although the Animal, Plant and Fisheries Quarantine and Inspection Agency (QIA) in Korea attempts to prevent the entry of all quarantined pathogens in imported plants, the identification of exotic plant pathogens and especially of viruses can be difficult because the host range of many plant viruses includes both known and unknown plant species. In addition, plant viruses may be present but may cause disease symptoms only under specific conditions. It is, therefore, important that quarantine agencies must have the ability to detect and identify plant viruses in both symptomatic and symptomless plants of all plant species.

In this study, we developed an improved method for the detection of five quarantine viruses in Korea: Beet black scorch virus (BBSV), Beet necrotic yellow vein virus (BNYVV), Eggplant mottled dwarf virus (EMDV), Pelargonium zonate spot virus (PZSV), and Rice yellow mottle virus (RYMV). Although these viruses have not been reported in Korea, they could infect economically important plants in this country if introduced. BBSV and BNYVV cause rhizomania disease of sugar beet (Jiang et al., 1999; Tamada and Kusume, 1991). Interestingly, both viruses are transmitted by fungal vectors; BBSV is transmitted in a non-persistent manner by zoospores of Olpidium brassicae (Jiang et al., 1999) while BNYVV is transmitted by the soil-borne fungus Polymyxa betae (Tamada and Kusume, 1991). BBSV is a member of the genus Necrovirus, and its genome consists of 3,641 bp that encode six proteins (Cao et al., 2002). BNYVV is a member of the genus Benyvirus and is composed of four or five RNA segments (Kiguchi et al., 1996; Saito et al., 1996). Although BBSV and BNYVV have not yet been reported in Korea, they have been reported in most other parts of the world (Cai et al., 1993; Chiba et al., 2011; González-Vázquez et al., 2009; Koenig and Valizadeh, 2008; Weiland et al., 2006). Mechanical inoculation demonstrated that BBSV has a wide host range and produces local lesions on Chenopodium amaranticolor, C. quinoa, C. murale, Spinacia oleracea, and Tetragnopia expansa (Cai et al., 1993). BBSV also latently infects Lactuca sativa, Physalis floriana, and Nicotiana species (Cai et al., 1993). Aside from sugar beet, BNYVV also infects various spinach cultivars (Mou et al., 2012). EMDV is a rhabdovirus, transmitted by a leafhopper (Babaie and Izadpanah, 2003) and infects many plants such as Capparis spinosa, cucumber, eggplant, tomato, Pittosporum tobira, potato, and tobacco (Katis et al., 2011; Mavrié et al., 2006). PZSV, belongs to the family Bromoviridae, was originally isolated from Pelargonium zonale and has a wide host range that includes tomato, globe artichoke, Capsella bursa-pastoris, Chrysanthemum segetum,
**Table 1.** The five quarantine plant viruses for which RT-PCR primers were designed in the current study

| Virus name                     | Family               | Genus       | Type                    | No. of RNA fragments | Host plants              |
|--------------------------------|----------------------|-------------|-------------------------|----------------------|--------------------------|
| Beet black scorch virus (BBSV) | Tombusviridae        | Necrovirus  | ssRNA positive-strand viruses | 1                    | Sugar beet, spinach, lettuce |
| Beet necrotic yellow vein virus (BNYVV) | Unclassified            | Benyvirus   | ssRNA positive-strand viruses | 5                    | Sugar beet, spinach |
| Eggplant mottled dwarf virus (EMDV) | Rhabdoviridae        | Nucleorhabdovirus | ssRNA negative-strand viruses | 1                    | Eggplant, Hibiscus rosasinensis, tomato |
| Pelargonium zonate spot virus (PZSV) | Bromoviridae        | Anulavirus  | ssRNA positive-strand viruses | 4                    | Tomato, globe artichoke, Capsella bursa-pastoris |
| Rice yellow mottle virus (RYMV)   | Unclassified         | Sobemovirus | ssRNA positive-strand viruses | 1                    | Rice (Oryza sativa and O. glaberrima) |
sets and the identical conditions described in the previous paragraph, all primer sets amplified PCR products of the expected sizes except for primer set RYMV_#3 (Fig. 2; lane 15). However, the quantities of amplified PCR products were less with EMDV_#2, EMDV_#3, and PZSV_#3 than with the other primer sets, indicating that annealing temperature or other conditions for RT-PCR amplification might not be optimal for these three primer sets.

To optimize annealing temperature for each primer set, we performed gradient PCRs with all 15 primer sets. The gradient PCRs were conducted with the following conditions: an initial denaturation at 94°C for 5 min; followed by 25 cycles at 94°C for 30 sec, a gradient annealing temperature (51.9, 54, or 55°C) for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 5 min. The results are shown in Fig. 3. With primer sets BBSV_#1 and BBSV_#3,
clear and substantial bands were obtained regardless of annealing temperature. With primer set BBSV_#2, however, the quantity of PCR product gradually increased as the annealing temperature increased. For BBYVV, PCR products were obtained with all primers at each annealing temperature tested but PCR bands were thicker at 55°C than at other temperatures. Expected bands were obtained with EMDV primer sets at all three annealing temperatures but the band was faint with primer set EMDV_#3 at 55°C. Among the three primer sets for PZSV, only PZSV_#1 amplified PCR products at all three temperatures. In contrast, primer sets PZSV_#2 and PZSV_#3 amplified PCR products only at 51.9°C. For RYMV, only primer set RYMV_#1 could amplify PCR products at all three tested temperatures. With primer set RYMV_#2, amplicons with faint bands were detected only at 54°C and 55°C. The primer set RYMV_#3 did not amplify any PCR product at the three temperatures, indicating that this primer set did not bind to the RYMV cDNA.

To be useful for quarantine purposes, the designed primer sets must not amplify sequences from other viruses or from the host genome. The specificity of 10 of the primer sets (BBSV_#1, BBSV_#3, BNYVV_#1, BNYVV_#3, EMDV_#1, EMDV_#2, PZSV_#1, PZSV_#2, RYMV_#1, and RYMV_#2) was tested using total RNA of tomato and tobacco and RNA obtained from Nicotiana benthamiana plants that had been inoculated with Potato virus X (PVX) or Cucumber mosaic virus (CMV) 7 days earlier. Total RNAs were extracted from the uninfected tomato and tobacco plants as well as the infected N. benthamiana by the Trizol method, and cDNAs were synthesized as described earlier. PCRs were performed with cDNAs of the five quarantine viruses that were the focus of this study (as positive controls), with the genomic DNAs of N. benthamiana and Solanum lycopersicum, and with the cDNAs derived from PVX- and CMV-infected N. benthamiana. PCRs with the 10 primer sets were performed as follows: an initial denaturation at 94.0°C for 5 min; followed by 25 cycles at 94.0°C for 30 sec, 50.0°C for 30 sec, and 72.0°C for 30 sec; and a final extension at 72.0°C for 5 min. PCR products were obtained only with the positive controls (Fig. 4). No band was detected in samples containing genomic DNA of tobacco or tomato, and no band was detected with the cDNAs derived from PVX- or CMV-infected plants. These results indicate that the designed RT-PCR primer sets are specific for the target viruses.

In summary, we designed 15 primer pairs for PCR detection of five viruses (BBSV, BNYVV, EMDV, PZSV and RYMV). These five viruses, which have relatively wide host ranges, have not been detected in Korea but would cause substantial economic losses if they were able to establish in Korea. Fourteen of the 15 primer pairs (all except RYMV_#3) amplified the expected PCR products. Gradient PCRs indicated that 51.9°C was the optimal temperature for the annealing temperature of the PCR reaction.

![Fig. 2. RT-PCR amplification of virus-specific dsDNA fragments using virus-specific primer pairs corresponding to the CP regions. The number above each lane indicates the primer set (and corresponding virus fragment) used in the RT-PCR as follows: lane 1, BBSV #1; lane 2, BBSV #2; lane 3, BBSV #3; lane 4, BNYVV #1; lane 5, BNYVV #2; lane 6, BNYVV #3; lane 7, EMDV #1; lane 8, EMDV #2; lane 9, EMDV #3; lane 10, PZSV #1; lane 11, PZSV #2; lane 12, PZSV #3; lane 13, RYMV #1; lane 14, RYMV #2; lane 15, RYMV #3. Lane M1 and M2 represent 1 kb molecular mass marker (Bioneer, Daejeon, Korea) and 100 bp DNA ladder (NEB, Herts, UK), respectively. Numbers on the sides of the gel indicate DNA sizes in kilobase pairs.](image1)

![Fig. 3. Gradient RT-PCRs using three primer sets and three annealing temperatures for each virus. For each virus, lanes 1–3, 4–6, and 7–9 were obtained with primer sets #1, #2, and #3, respectively. The annealing temperature used to obtain the products for each primer set was: 51.9°C for lanes 1, 4, and 7; 54.0°C for lanes 2, 5, and 8; and 55.0°C for lanes 3, 6, and 9. Lane M indicates 1 kb molecular mass marker (Bioneer, Daejeon, Korea). Numbers located to the left of the gel indicate DNA sizes in kilobase pairs.](image2)
Specific Detection of Five Exotic Quarantine Plant Viruses

14 primer sets. Primer pairs BBSV #1 and BBSV #3 performed well regardless of annealing temperature while primer pairs PZSV #2 and PZSV #3 required a specific annealing temperature. A primer specificity test using plant genomic DNAs and cDNAs derived from the other plant viruses indicated that the primer sets were specific for the five target viruses. Taken together, the results indicate that the primer sets described here will be useful for detecting BBSV, BNYVV, EMDV, PZSV, and RYMV and preventing their entry into Korea.

Acknowledgments

This research was supported in part by grants from the Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries (No. 309015-04) and from the Rural Development Administration (Nos. PJ00819801 & PJ007412). HC, WKC and JY were supported by the fellowships from the MEST through Brain Korea 21 Project.

References

Allarangaye, M., Traoré, O., Traoré, E., Millogo, R., Guinko, S. and Konate, G. 2007. Host range of Rice yellow mottle virus in Sudano-Sahelian Savannas. Pakistan J. Biol. Sci. 10:1414.

Bakker, W. 1974. Characterization and ecological aspects of Rice yellow mottle virus in Kenya. Agric. Res. Rep. 829.

Cai, Z., Chen, D., Wu, M., Cui, X., Yu, J. and Liu, Y. 1993. Identification of pathogenic virus of beet black scorch disease and detection by synthesized cDNA probes. J. Beijing Agric. Univ. 19:112.

Cao, Y., Cai, Z., Ding, Q., Li, D., Han, C., Yu, J. and Liu, Y. 2002. The complete nucleotide sequence of Beet black scorch virus (BBSV), a new member of the genus Necrovirus. Arch. Virol. 147:2431–2435.

Chiba, S., Kondo, H., Miyanishi, M., Andika, I. B., Han, C. and Tamada, T. 2011. The evolutionary history of Beet necrotic yellow vein virus deduced from genetic variation, geographical origin and spread, and the breaking of host resistance. Mol. Plant-Microbe Interact. 24:207–218.

Finetti-Sialer, M. and Gallitelli, D. 2003. Complete nucleotide sequence of Pelargonium zonate spot virus and its relationship with the family Bromoviridae. J. Gen. Virol. 84:3143–3151.

González-Vázquez, M., Ayala, J., García-Arenal, F. and Fraile, A. 2009. Occurrence of Beet black scorch virus infecting sugar beet in Europe. Plant Dis. 93:21–24.

Hebrard, E., Fargette, D., Konate, F. G., Faso, B. 2009. Rice yellow mottle virus. Desk Encyclopedia Plant Fungal Virol. 88:302.

Jiang, J., Zhang, J., Che, S., Yang, D., Yu, J., Cai, Z. and Liu, Y. 1999. Transmission of Beet black scorch virus by Olpidium brassicae. J. Jiangxi Agric. Univ. 21:525–528.

Fig. 4. Specificity of 10 of the newly designed primers for detection of BBSV, BNYVV, EMDV, PZSV, and RYMV by RT-PCR. Two primer pairs were tested for each quarantine virus. Amplified dsDNA fragments were separated in a 1% agarose gel and stained with EtBr. Pc represents the “positive control”, the amplified dsDNA fragments for the target virus. Nb and Ly represent amplified dsDNA fragments corresponding to healthy Nicotiana benthamiana and healthy Lycopersicon esculentum, respectively. PV and CM represent amplified dsDNA fragments using total RNAs extracted from N. benthamiana infected with PVX and CMV, respectively. Lane M indicates lambda DNA digested by HindIII as a molecular mass size marker. Numbers located to the left indicate sizes of digested lambda DNA in kilobase pairs.
Katis, N. I., Chatzivassiliou, E. K., Clay, C. M., Maliogka, V. I., Pappi, P., Efthimiou, K., Dovas, C. I. and Avgelis, A. D. 2011. Development of an IC-RT-PCR assay for the detection of Eggplant mottled dwarf virus and partial characterization of isolates from various hosts in Greece. *J. Plant Pathol.* 93:353–362.

Kiguchi, T., Saito, M. and Tamada, T. 1996. Nucleotide sequence analysis of RNA-5 of five isolates of Beet necrotic yellow vein virus and the identity of a deletion mutant. *J. Gen. Virol.* 77:575–580.

Koenig, R. and Valizadeh, J. 2008. Molecular and serological characterization of an Iranian isolate of Beet black scorch virus. *Arch. Virol.* 153:1397–1400.

Lee, B. Y., Lim, H. R., Choi, J. Y. and Ryu, K. H. 2004. Development of molecular detection of three species of seed-transmissible viruses useful for plant quarantine. *Plant Pathol. J.* 20:302–307.

Lee, J. S., Cho, W. K., Choi, H. S. and Kim, K. H. 2011a. RT-PCR detection of five quarantine plant RNA viruses belonging to poty- and tospoviruses. *Plant Pathol. J.* 27:291–296.

Lee, J. S., Cho, W. K., Lee, S. H., Choi, H. S. and Kim, K.-H. 2011b. Development of RT-PCR based method for detecting five non-reported quarantine plant viruses infecting the family Cucurbitaceae or Solanaceae. *Plant Pathol. J.* 27:93–97.

Mavriè, I., Tušek Žnidariè, M., Viršèek Marn, M., Dolnièar, P., Mehle, N., Lesemann, D. E. and Ravnikar, M. 2006. First report of Eggplant mottled dwarf virus in potato and tomato in Slovenia. *Plant Pathol.* 55:566–566.

Mou, B., Richardson, K., Benzen, S. and Liu, H. Y. 2012. Effects of Beet necrotic yellow vein virus in spinach cultivars. *Plant Dis.* 96:618–622.

Quacquarelli, A. and Gallitelli, D. 1979. Three viruses of Pelargonium zonale in Apulia. *Phytopathol. Mediterr.* 18:61–70.

Saito, M., Kiguchi, T., Kusume, T. and Tamada, T. 1996. Complete nucleotide sequence of the Japanese isolate S of Beet necrotic yellow vein virus RNA and comparison with European isolates. *Arch. Virol.* 141:2163–2175.

Tamada, T. and Kusume, T. 1991. Evidence that the 75K readthrough protein of Beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus Polymyxa betae. *J. Gen. Virol.* 72:1497–1504.

Weiland, J. J., Larson, R. L., Freeman, T. P. and Edwards, M. C. 2006. First report of Beet black scorch virus in the United States. *Plant Dis.* 90:828.

Yassi, M. N. A., Ritzenthaler, C., Brugidou, C., Fauquet, C. and Beachy, R. N. 1994. Nucleotide sequence and genome characterization of Rice yellow mosaic virus RNA. *J. Gen. Virol.* 75:249–257.