Plant Pathol. J. 22(3) : 255-259 (2006)

The Plant Pathology Journal

The Pear Black Necrotic Leaf Spot Disease Virus Transmitted by *Talaromyces flavus* Displays Pathogenicity Similar to *Apple stem grooving virus* Strains

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(Received on August 1, 2006; Accepted on August 16, 2006)

The pathogenicity to pear trees and other experimental hosts of the *Apple stem grooving virus* Korean isolate (ASGV-K) carried by a fungal vector, *Talaromyces flavus* was examined. ASGV-harboring *T. flavus* induced mild symptoms on virus-free pears. Symptom severity was intermediate between pears showing typical PBNLS and virus-free pears. Ten cultivars of *Phaseolus vulgaris* showed 35%-90% infectivity by direct infiltration into leaves and roots by ASGV-harboring *T. flavus*. Application of fungal cultures to soils showed 0%-70% infectivity depending on the *P. vulgaris* cultivar. Sap extracted from ASGV-infected *Chenopodium quinoa* induced similar symptoms on *P. vulgaris* at 25 days after inoculation. Similar symptoms were also detected on *P. vulgaris* which were inoculated with ASGV-harboring *T. flavus*. When healthy *P. vulgaris* leaves were challenged with sap extracted from *P. vulgaris* leaves infected with ASGV-harboring *T. flavus*, typical symptoms were observed. These data suggest that *T. flavus* mediates the transfer of ASGV to host plants.

Keywords **:** *Apple stem grooving virus*, pear black necrotic leaf spot, *Phaseolus vulgaris*, *Talaromyces flavus*

Recently, we reported that pear black necrotic leaf spot (PBNLS) could be caused by *Apple stem grooving virus* (ASGV) (Shim et al., 2004). However, there was no information about the viral vectors and transmission manner of ASGV on pears.

Twenty plant viruses are currently known to be transmitted by fungal zoospores, and, in many cases, this is their primary or only means of transmission (Adams, 1991). This is a testament to the efficiency of zoospores in locating a host. However, the vectors belong to just three genera, *Olpidium* (Chytridiomycota), and *Polymyxa* and *Spongospora* (plasmodiophorids). These fungi are common and usually symptomless parasites of roots, and their significant feature as viral vectors is that the encysted zoospores germinate to release a naked protoplast into the plant (Alexopoulos et al., 1996). Fungi (Oomycota) that produce walled hyphae from zoospore cysts appear to be incapable of acting as viral vectors.

Contrary to these studies, von Wechmar et al. (1992) have claimed that three viruses are transmitted by higher fungi (Erasnus et al., 1983). They observed that uredospores of *P. graminis tritici* (stemrust of wheat) transmitted *Brome mosaic virus* (BMV) when uredospores developed on plants that were simultaneously infected with stemrust and BMV (von Wechmar, 1980; Erasmus and von Wechmar, 1983). Additionally, they showed that transmission of *Maize dwarf mosaic virus* (MDMV) to new maize seedlings by uredospores was maintained for three successive years (1988-1991) in a plant growth room.

Talaromyces flavus is considered a member of the higher fungi. *T. flavus* (anamorph: *Penicillium vermiculatum*) is an ascomycetous fungus, and is the most common species of *Talaromyces*, which is found throughout the world in soils of warmer climates (Murray et al., 1997). It first attracted attention as a parasite of *Rhizoctonia solani* because it was found to coil around the hyphae on agar plates, penetrating them and causing localized disruption. However, recent interest has focused on its potential to control the vascular wilt pathogen, *Verticillium dahliae* (Marois et al., 1982). *T. flavus* invades the melanized microsclerotia of *V. dahliae* on diseased roots and sporulates on the surfaces of these structures. *T. flavus* has often been found in soil, but the

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pathogenicity of *T. flavus* and its ability to act as a virus transmitting vector have not been reported previously. A few studies have suggested that higher fungi such as *T. flavus* deliver plant viruses, but more definitive studies have not appeared.

Here, in order to investigate why and how the number of PBNLS disease was increased in field we show that ASGV could be transmitted by *T. flavus* as a putative vector. We also developed an experimental host system to induce symptoms and investigated the transmissibility of ASGV by inoculating host plants with ASGV-harboring fungi.

Materials and Methods

Seeds. *C. quinoa* seeds were obtained from the National Horticultural Research Institute. *P. vulgaris* seeds were obtained from the National Seed Management Office. The ten cultivars of *Phaseolus vulgaris* used in this study were Kyunggi 1, Kyunggi 2, Kyunggi 3, Kyunggi 4, Kyunggi 5, Kangnangkong 1, KGP 6029, KGP 7059, Chungye 20, Chyungye 23, and Sansung.

Fungal transmission tests. Isolates of virus-free and virus-infected *T. flavus* were grown on potato dextrose agar (PDA) for 14 days. Conidial suspensions were prepared in distilled water containing 0.01% Tween-20. *P. vulgaris*, *C. quinoa,* and *N. tabacum* seedlings were used as test plants for viral transmission. Soils were autoclaved at 121°C for 15 min and dried at 65°C. The seedlings were supplied with modified Hoagland and Arnon solution (pH 7.0) daily, and were cultured in a growth chamber at 25°C under a 16 h light/8 h dark cycle (Tamada et al., 1989). When seedlings reached the 4-6 leaf stage, plant leaves were inoculated with fungus, or liquid fungal cultures were applied to the soil. Leaves were given a wound of approximately 1 mm in length with a sterilized scalpel and were inoculated with 10 μL of conidial suspension (1×10^6 conidia/mL) or a 0.5×0.5 mm mycelial block. Inoculations were performed with 3 replicates per leaf. Inoculated plants were kept in a plant growth room with high relative humidity (approx. 70%) maintained by an automatic humidifier. To prepare an inoculum for soil applications, 10 μL of conidial suspension (1×10^6 conidia/mL) was introduced into 250 mL of PDB and incubated for 14 days.

RT-PCR. To determine the possible transmission of ASGV by *T. flavus*, RT-PCR was carried out on fungi and inoculated plants. A single cycle (consisting of 10 min at 70°C, 50 min at 42°C, 5 min at 95°C) was used to reverse transcribe 5 μg of RNA treated with RQ-1 DNase (Promega) in a reaction volume of 30 μL (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5.5 mM $MgCl_2$, 500 μM each dNTP, 0.4 units RNase inhibitor/μL, 1.25 units reverse transcriptase/μL). The resulting cDNA (5 μL) was then subjected to PCR amplification (5 min at 93°C, followed by 30 cycles of 20 s at 94°C, 40 s at 57°C, 40 s at 72°C) using Hi-Fidelity PCR amplification reagents (Roche). Primers for amplification of ASGV were designed based on the nucleotide sequence of the reported ASGV (AF465354). The primer pairs used for the amplification of the coat protein (CP) were: ASGV-P1, 5'-CAT ATG AGT TTG GAA GAC GTG CTT C-3' and ASGV-P2, 5'-CTC GAG ACC CTC CAG TTC CAA GTT A-3'. The resulting PCR product was subcloned into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions.

Dot-blot hybridization. Dot-blot hybridization was used to detect ASGV in plants and fungi. The quality of the RNA was checked on a 1% agarose gel after denaturation for 10 min at 70°C in 5 M urea. The RNA concentration and purity were determined by UV absorbance. RNA samples (100 ng) from soil samples and from pure fungal cultures were denatured in three volumes of 2% gluteraldehyde in 50 mM phosphate buffer (pH 7.0) and applied in duplicate to Hybond-N+ positively-charged membranes (Amersham Pharmacia Biotech) using a 96-well Miniford I dot-blot system (Midwest Scientific) under vacuum pressure. The membranes were air-dried prior to hybridization. A [^{32}P]dCTP-labeled pASCP clone was used as the probe. Unincorporated nucleotides were removed with a nucleic acid removal kit (Qiagen), according to the manufacturer's instructions. Membranes were pre-hybridized in Perfect Hyb Plus hybridization buffer (Amersham Pharmacia Biotech) for 15 min at 42°C before adding the labeled probe. After overnight hybridization, membranes were washed twice in 100 mL of a wash solution consisting of 1x SSC (0.15 M NaCl, 0.015 M sodium citrate) and 1% sodium dodecyl sulfate at 42°C. Each membrane was then washed for 30 min in 200 mL of the wash solution at the specified wash temperature for each probe. After washing, the membranes were air-dried and exposed to film for 2 days at -70°C.

Results and Discussion

To compare PBNLS symptoms with those of pears infected with ASGV-harboring *T. flavus*, three pear tree treatments were used. Ten scions from healthy plants were top-grafted onto healthy rootstocks as controls in this study. Two experimental groups consisted of ten healthy scions top-grafted onto plants that had shown signs of PBNLS, and ten healthy plants that were inoculated with ASGV-harboring *T. flavus*.

PBNLS-like symptoms developed in the experimental plants in six months after grafting (Table 1). Necrotic spots

Table 1. Infection rates of pear trees inoculated with ASGV-harboring fungal isolates and top-grafting

Methods	N[a]	% infected[b]	severity[c]	DPI[d]	RT-PCR
Top grafting (Healthy plants)	10	0	0	–	–
Top grafting (Indicator plants)	10	70	3-5	6 months	+
Virus-infected fungus Inoculation	10	20	2	6 months	+

[a]number of plants treated
[b](number of plants showing symptoms/total number of plants treated) × 100
[c]degree of symptom development (0; no symptoms, 1; mild symptoms, 2; moderate symptoms, and 3; severe symptoms)
[d]DPI (days post-inoculation)

were produced on the leaves of seven out of ten scions grafted onto PBNLS infected plants. Typical symptoms were observed on leaves of the top-grafted scions and were used as positive controls (Fig. 1D). PBNLS-like symptoms also developed in plants infiltrated with ASGV-harboring *T. flavus*. The symptoms were similar to those of the grafted plants, but were relatively mild overall (Fig. 1B). In the case of the PBNLS infected plants, necrotic spots became much larger due to fusion of the lesions (Fig. 1D). None of virus-free fungi infected pears or virus-free scions of pears developed symptoms (Fig. 1A, 1C). These data indicated that ASGV can be transmitted both by grafting and ASGV-harboring *T. flavus*. This was the first report about the ASGV transmission by fungus, not by grafting.

RT-PCR was used to detect the ASGV CP gene and confirmed the presence of the virus. RT-PCR using CP gene specific primers was performed on total RNA extracts and a PCR product of the expected size (714 bp) was amplified from plants grafted onto PBNLS infected plants, but not from healthy plants (data not shown). Symptomatic leaves of plants inoculated with ASGV-harboring *T. flavus* also produced a 714 bp PCR product. The PCR products were sequenced and compared with ASGV (AF465354). These sequences showed extremely high homology (98%) to the ASGV CP sequence in the database (data not shown).

Fig. 1. Symptoms caused by virus-infected fungi and grafting. (A) Virus-free *T. flavus*-infected pear, (B) virus-infected *T. flavus*-inoculated pear, (C) Virus-free pear tree, (D) Virus-infected indicator plants.

To study the complex interactions among host plant, fungus, and virus, we used the experimental hosts *P. vulgaris* and *C. quinoa* which are susceptible to ASGV as well as to virus-infected fungi. *N. tabacum* was also tested to determine whether it could be used as another experimental host (Table 2). *N. tobacum* was asymptomatic to ASGV-harboring *T. flavus*. In this study, two inoculation methods were applied for investigating which organs could be the possible route for infection by ASGV-harboring *T. flavus*: (A) mechanical inoculation of fungi onto the leaves and (B) direct application of suspension-cultured mycelia to the soil. Ten cultivars of *P. vulgaris* showed 35%-90% infectivity when they were inoculated by direct inoculation into wounds on the leaves (Table 2A). Direct application of fungal cultures to the soil produced infection rates of 0%-70% among the different *P. vulgaris* cultivars (Table 2B). Infectivity varied depending on the method of inoculation and the *P. vulgaris* cultivar, due to specific interactions between *P. vulgaris*, ASGV, and the ASGV-harboring fungus. The Kyunggi 5 cultivar showed the highest rates of infection under either method of inoculation and, thus, should be a suitable experimental host for future analyses. When leaves of two other herbaceous hosts, *C. quinoa* and *N. tabacum*, were inoculated, or when liquid cultures of fungus were applied to the soil surrounding the plants, the plants were not infected to the same degree as *P. vulgaris* (Table 2). *C. quinoa* showed 21% infectivity when fungal cultures were applied to the soil surrounding the roots, but the disease symptoms were not clear and a relatively long amount of time was needed for symptoms to appear. *N. tabacum* was not infected by ASGV regardless of inoculation method. In all cases, inoculation of leaves induced disease symptoms faster than inoculation of roots by application of fungal cultures to the soil.

Table 2. Infection rates of various *P. vulgaris* cultivars inoculated with ASGV-harboring fungal isolate

(A) Inoculation of fungus onto the leaves

Plants	N[a]	% infected[b]	severity[c]	DPI[d]	RT-PCR
Phaseolus vulgaris					
Kyunggi 1	20	85	4	10	+
Kyunggi 2	20	85	3	17	+
Kyunggi 4	20	85	4	12	+
Kyunggi 5	20	90	5	10	+
Kangnangkong 1	20	50	2	17	+
KGP 6029	20	75	3	11	+
KGP 7059	20	75	3	14	+
Chungkye 20	20	50	2	20	+
Chungkye 23	20	50	2	20	+
Sansung	20	35	1	23	+
Chenopodium quinoa	14	0	0	ND[e]	ND
Nicotiana tabacum	14	0	0	ND	ND

[a]number of plants treated
[b](number of plants showing symptoms/total number of plants treated) × 100
[c]degree of symptom development (0; no symptoms, 1; mild symptoms, 2; moderate symptoms, and 3; severe symptoms)
[d]DPI (days post-inoculation)
[e]Not determined

(B) Application of fungal culture to the soil.

Plants	N[a]	% infected[b]	severity[c]	DPI[d].	RT-PCR
Phaseolus vulgaris					
Kyunggi 1	10	50	2	14	+
Kyunggi 2	10	50	1	20	+
Kyunggi 4	10	50	2	16	+
Kyunggi 5	10	70	3	14	+
Kangnangkong 1	10	20	1	19	+
KGP 6029	10	40	2	16	+
KGP 7059	10	40	1	19	+
Chungkye 20	10	ND[e]	0	ND	+
Chungkye 23	10	ND	0	ND	+
Sansung	10	0	0	ND	ND
Chenopodium quinoa	14	21	1	20	+
Nicotiana tabacum	14	0	0	ND	ND

[a]number of plants treated
[b](number of plants showing symptoms/total number of plants treated) × 100
[c]degree of symptom development (0; no symptoms, 1; mild symptoms, 2; moderate symptoms, and 3; severe symptoms)
[d]DPI (days post-inoculation)
[e]Not determined

Sap extracted from ASGV-infected *C. quinoa* induced similar symptoms on *P. vulgaris* at 25 days after inoculation (Fig. 2B). Symptoms were also detected in *P. vulgaris* treated with ASGV-harboring *T. flavus* (Fig. 2D). When sap extracted from *P. vulgaris* leaves infected with ASGV-harboring *T. flavus* was used to inoculate new healthy *P. vulgaris* leaves, similar symptoms were observed (Fig. 2F). However, *P. vulgaris* inoculated with mock treatment (Fig. 2A), ASGV-free *T. flavus* (Fig. 2C), or saps from ASGV-free *T. flavus* infected *P. vulgaris* (Fig. 2E) did not show any disease symptoms. Collectively, with the data of Fig. 2, we concluded that (1) ASGV can be transmitted by *T. flavus* and (2) the symptoms shown on *P. vulgaris*, pear, and *C. quinoa* (data not shown) were induced by ASGV-harboring *T. flavus*, not by *T. flavus* only.

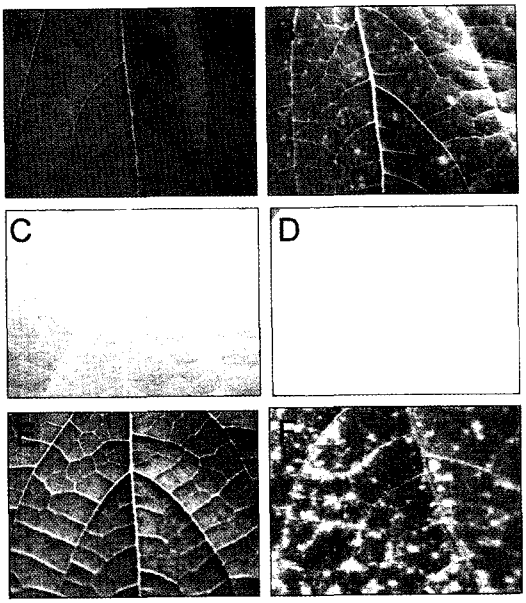

Fig. 2. Comparison of symptoms on leaves of *Phaseolus vulgaris* at 25 dpi. The leaves on the left are buffer-inoculated leaves (A), (C) and (E) which served as the negative controls, and the leaves on the right were inoculated with (B) infectious ASGV plant sap, (D) ASGV-harboring *T. flavus*, and (F) plant sap from ASGV-harboring *T. flavus* infected leaves.

The plant samples shown in Fig. 2 were used for RNA isolation, and 5 μg RNA was used for dot-blot hybridization to detect the presence of the virus (Fig. 3). ASGV was not detected in plants treated with the virus-free fungal isolate or mock-inoculated (Fig. 3). However, ASGV was detected in all symptomatic leaves (Fig. 3). Taken together, these data indicated that *T. flavus* mediates the transfer of ASGV into host plants by ASGV harboring *T. flavus* infiltration. With limited information and data about virus transmission by fungus such as *T. flavus*, we could not explain the mechanism of ASGV transmission 'from plant to plant' and 'from fungus to plant'. But the challenging of saps from ASGV infected *C. quinoa* on *P. vulgaris*, infiltration of ASGV harboring *T. flavus* on *P. vulgaris* and re-inoculation of saps from ASGV harboring *T. flavus* infiltrated leaves showed the symptom induction and

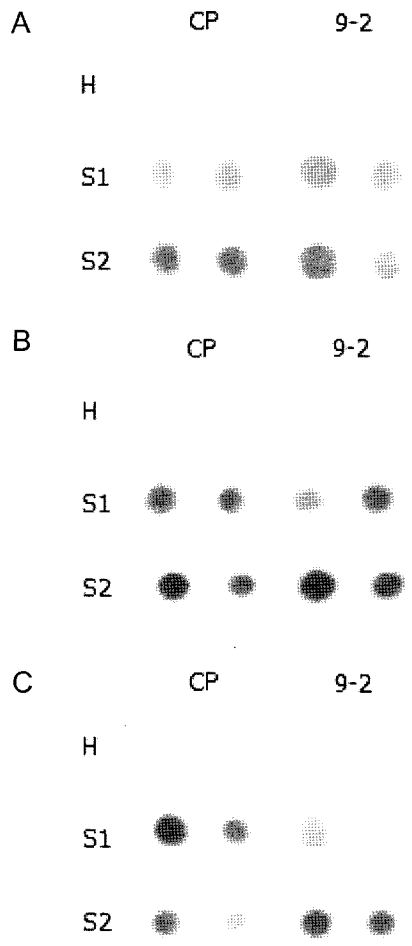

Fig. 3. Dot-blot hybridization of total RNA extracted from *Phaseolus vulgaris*. (A) Total RNA extracted from symptomatic leaves treated with plant sap from ASGV infected plants; (B) total RNA extracted from *P. vulgaris* inoculated with ASGV-harboring *T. flavus*; (C) total RNA extracted from *P. vulgaris* inoculated with plant sap from leaves inoculated with ASGV-harboring *T. flavus*. Duplicate membranes were hybridized with specific probes for the coat protein (CP) region and the N-terminal (9-2) region of the viral genome. H indicates a healthy plant. S1 and S2 are two independent symptomatic plants.

ASGV accumulation in all three trials, demonstrating that somehow ASGV could be transmitted by ASGV harboring *T. flavus*.

Acknowledgements

This study was supported by a grant from the Agricultural Research Promotion Center (203051-3), Ministry of Agriculture and Forestry, Republic of Korea.

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