A Nested PCR Assay for Detecting *Valsa mali* var. *mali* in Different Tissues of Apple Trees

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Abstract

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A nested polymerase chain reaction (PCR) assay for detecting *Valsa mali* var. *mali*, the causal agent of apple tree Valsa canker, was developed. One pair of genus-specific primers was designed based on the ribosomal DNA internal transcribed spacer conservative sequence of the *Valsa* genus and one pair of species-specific primers was designed based on the specific sequence of *V. mali* var. *mali*. The specificity of the genus-specific and species-specific primers was evaluated against 10 *V. mali* var. *mali* isolates, 10 *V. mali* var. *pyri* isolates, 4 isolates from closely related *Valsa* spp., and 8 isolates from fungal species that are commonly isolated from naturally infected apple bark tissue. A distinct band of 348 bp in length was detected in all *V. mali* var. *mali* isolates but not in other tested species and the *V. mali* var. *pyri* variety. The sensitivity of this assay was evaluated by serial dilutions of DNA extracted from *V. mali* var. *mali* pure cultures and apple bark tissues with or without visible symptoms. The results showed that the assay was able to detect as little as 100 fg of DNA in mycelial samples and apple bark tissues with visible symptoms, whereas the lowest detecta-

ble concentration was 10 pg of DNA in symptomless apple bark tissues. The efficiency of the nested PCR assay was compared with that of fungal isolation assays. All symptomless and symptomatic samples from which the pathogen was successfully isolated yielded a PCR product of the expected size. The detection rate of nested PCR for symptomless samples was 64.7%, which was much higher than the detection rate of 20.6% by fungal isolation. The PCR analysis of different symptomless tissues showed that the incidence of *V. mali* var. *mali* was different in different tissues of apple trees. The average incidence of *V. mali* var. *mali* was 89% in terminal buds, 71% in internodes, and 48% in bud scale scars. Moreover, the incidence of *V. mali* var. *mali* in nonsymptomatic tissues was higher in orchards where more trees were infected. Taken together, the assay developed in this study can be used for rapid and reliable detection of *V. mali* var. *mali* in tissues of apple trees with or without symptoms and also for monitoring the presence of the pathogen at an early stage of disease development.

Valsa canker, caused by *Valsa mali* Miyabe & Yamada, is one of the most destructive stem diseases of apple trees (*Malus* spp.) in China (4,18,19), Japan (1), and Korea (15) and causes serious yield losses in apple-producing regions, especially in Shaanxi province (16,21). An investigation in 2003 and 2004 in Shaanxi province showed, on average, 30% diseased trees; whereas, in some orchards, the incidence of diseased trees was over 90% (16). Because little is known about the genetic characteristics of the pathogen, an effective disease control strategy was not established for a long time. Recently, it was reported that significant intraspecific genetic differentiation exists in *V. mali*; two varieties, *V. mali* var. *mali* and *V. mali* var. *pyri*, were recognized (18). *V. mali* var. *mali* just infects apple, whereas *V. mali* var. *pyri* can occur on both apple and pear. However, the two varieties have different pathogenicity on apple. *V. mali* var. *mali* was significantly more aggressive on apple than *V. mali* var. *pyri* (18).

The disease is characterized by cankers on trunks, twigs, and scaffold limbs (14). Cankers are sunken, swollen, distorted, and cracked areas of bark that may be dotted with red pustules. A large number of black pycnidia appear on the surface of cankers (13). Importantly, the infection always leads to expanding cankers, resulting in the death of twigs, branches, trunks, and eventually the entire tree (1). Entire orchards destroyed by this disease are prevalent in many apple-producing regions in China (19,21).

The pathogen infects trees through damage or injury to the bark, such as fresh pruning wounds, fruit scars, and freeze injury (13). The typical symptoms are easy to observe in winter and spring. The fruiting bodies and spores are readily found in spring. *Cytospora* spp., the anamorphs of *Valsa* spp., have been considered facultative wound parasites of many broadleaf tree species (2). Christensen et al. (5) suggested that *Cytospora* spp. might cause symptomless infection as a latent pathogen. In most cases, the latent period will last until the host becomes stressed. However, it remains unknown whether the asymptomatic sound bark and other tissues of apple trees carry the causal fungi or not.

It is very important to address this critical issue in order to obtain more complete knowledge of the latent infection and disease cycle, and implement an effective strategy for controlling this disease. There are two methods that can be used to detect this pathogen in apple tree tissue: either by direct isolation of the pathogen or by molecular detection techniques. However, the isolation of *Valsa* spp. in newly infected tissues as well as latent infection of apparently healthy tissue by conventional methods is difficult and time consuming.

With the development of molecular techniques based on DNA analysis, the polymerase chain reaction (PCR) method has become one of the most effective and dependable methods for detection, identification, and classification of plant-pathogenic fungi (10). The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) genes are ideal candidates for designing species-specific primers because they are conserved within the same species but variable between different species. Furthermore, the rDNA gene sequence appears in multiple copies in the genome, resulting in more efficient PCR amplification and, thereby, a higher sensitivity for detection (6). The species-specific primers based on the rDNA-ITS sequence in the PCR have been frequently applied to detection and identification of many different pathogens in plants (6,11,12).

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However, because PCR inhibitors, such as phenolic compounds, are often present in woody plant tissues, conventional one-step PCR techniques are not suitable to identify and detect pathogens in these crops. Thus, a more sensitive PCR assay is needed. The nested PCR appears to be a logical choice because of its increased sensitivity and specificity of detection (17). This assay has been successfully applied for detection of fungal pathogens in woody plants. *Eutypa lata* and *Eutypella vitis* were specifically detected in grapevine as well as latent infections by *Sphaeropsis sapinea* in Austrian pine tissue (3,6). In light of these results, the nested PCR assay has a potential to be routinely used for detecting pathogens in plant tissues.

The objectives of this study were to (i) develop a reliable and sensitive PCR-based method for detecting and identifying *V. mali* var. *mali* in symptomatic and symptomless infected apple tissues and (ii) determine the distribution of this pathogen in different apple tree tissues.

Materials and Methods

Fungal isolation. *V. mali* var. *mali* isolates used in this study were partly provided by the Integrated Plant Diseases Management Laboratory in the College of Plant Protection, Northwest A&F University, and some isolates were obtained from infected apple tree bark of limbs from different orchards in Shaanxi province. The infected twigs with visible canker symptoms were collected in plastic bags and, upon arrival in the laboratory, were washed with tap water and air dried at room temperature. Between the symptomless and apparently infected area, samples were taken and cut into small pieces, which were soaked into 70% ethanol for 2 to 3 min. Then, the pieces were washed twice with sterile water to remove the ethanol from the surface of the samples and transferred with sterile forceps onto potato dextrose agar (PDA) in petri dishes. The plates were incubated at 25°C for 3 days, and mycelial plugs were cut with a cork borer from the margins of the colonies and transferred to water agar plates. After incubation at 25°C for 5 days, single hyphal tips were cut with a sterile scalpel under the microscope and transferred to PDA to obtain a single hyphal colony. Stock cultures of all single hyphal isolates were stored in 15% glycerol at -80°C.

DNA extraction from the single hyphae cultures. For DNA extraction, the stock cultures were transferred onto PDA plates and incubated at 25°C for 3 days in the dark and, from the edge of the colony, five small plugs (5 mm in diameter) were transferred into 25 ml of potato dextrose broth in 250-ml flasks. The cultures were incubated as still culture at 25°C for 5 days. The mycelium was filtered through sterile gauze and approximately 250 mg of fresh mycelium was collected into 2.0-ml microcentrifuge tubes and then lyophilized for DNA extraction. The procedure for DNA extraction followed that described by Lee et al. (7), with slight modifications. The lyophilized mycelium was ground to a fine powder in a mortar and pestle, and then the powder was suspended in 800 µl of lysis buffer (50 mM ethylene diamine tetraacetic acid [EDTA], 100 mM Tris-HCl [pH 8.0], and 3% sodium dodecyl sulfate [SDS]), followed by brief vortexing and then incubation at 65°C for 1.5 h. Subsequently, 800 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the mixture. The tubes were gently inverted several times and centrifuged at room temperature for 15 min at 12,000 rpm. The aqueous phase was transferred to a 1.5-ml tube and an equal volume of chloroform was added to the tube. The mixture was centrifuged again at the same speed, the top aqueous layer was transferred into a 1.5-ml tube, and 10 µl of 3 M sodium acetate and 200 µl of isopropanol were added. After inverting the tubes several times, threads of precipitated DNA were observed. The suspension was centrifuged at 12,000 rpm for 10 min, the supernatant was poured off carefully, and the DNA pellet was rinsed twice with 100 µl of 70% ice-cold ethanol. When the ethanol had almost evaporated, the pellet was suspended in 50 µl of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) overnight at 4°C. Afterward, 1.0 µl of RNase at 10 mg/ml (Sigma-Aldrich) was added to digest RNA in the DNA suspension, which

was incubated at 37°C for 2 h. After evaluation of the quality of DNA by an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.), the DNA was stored at -20°C.

Collection of symptomatic and symptomless tissue samples.

In order to know the relationship between the diseased tree incidence and *V. mali* var. *mali* incidence in symptomless tissue in apple orchards, the symptomless samples used for detection of *V. mali* var. *mali* were collected from six orchards in Yangling, Shaanxi province. The incidence of diseased trees in each orchard was calculated by evaluation of 25 trees. In each orchard, five trees with typical symptoms were then randomly selected and four 1-year-old symptomless twigs in four directions were cut from each tree. All samples originating from the same orchard were collected in a plastic bag. The samples were washed with tap water and air dried in the laboratory. The terminal buds and bud scale scars of each twig were collected separately. The internode tissues of each twig were selected randomly. Twigs showing visible pycnidia or water-soaked cankers were also collected. Then, the buds and bark and canker tissues were cut into small pieces and placed in an autoclaved mortar. Liquid nitrogen and quartz sand were added and the samples were ground to a fine powder with a pestle. The powder was transferred into a 2.0-ml microcentrifuge tube and used for DNA extraction.

Fungal DNA extraction from symptomless apple tissues. The fungal DNA from symptomless apple twigs was extracted as described by Zhang et al. (22), with slight modifications. The powder samples were suspended in 800 µl of extraction buffer, containing 2% (wt/vol) cetyltrimethylammonium bromide (CTAB), polyvinyl pyrrolidone (PVP), β-mercaptoethanol, 1.4 M NaCl, 20 mM EDTA (pH 8.0), and 100 mM Tris-HCl (pH 8.0), and incubated at 65°C for 2 h followed with gentle shaking every 10 min. Then, an equal volume (800 µl) of phenol/chloroform/isoamyl alcohol (25:24:1) was added to each tube. The tubes were inverted several times and centrifuged at 12,000 rpm for 10 min. The supernatant (500 µl) was transferred to a 1.5-ml tube and eluted with an equal volume of chloroform. After centrifugation (at 12,000 rpm for 10 min), the aqueous phase (350 µl) was collected and DNA was precipitated by addition of 60 µl of 3M sodium acetate and a two-thirds volume of isopropanol followed by incubation at -20°C overnight. The following procedure is the same as the extraction of DNA from mycelium described above.

ITS amplification, sequencing, and primer design. The ITS region of the pathogen was amplified by the universal primers designated as ITS1 (5'-TCC GTA GGT GAA CCT GCG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT-3') to assess the suitability of the DNA isolated for amplification (20). The PCR assay conditions were the same as those described by Wang et al. (18). The PCR products were sequenced along both strands with ITS1 and ITS4 primers using an Applied Biosystems Sequencer 3130XL (Applied Biosystems). The ITS sequences for different *Valsa* spp., including *V. malicola* Z. Urb (DQ243802), *V. persoonii* Nitschke (DQ996042), *V. sordida* Nitschke (EF101159), *V. ambiens* (Pers.) Fr. (AY347339), and *V. salicina* (Pers.) Fr. (EF447417), were publicly available on the National Center for Biotechnology Information website. These sequences were analyzed using EditSeq and SeqMan in the DNASTAR software (DNASTAR, Inc.). The conserved ITS sequences within the genus *Valsa* were used to design the first pair of primers by the software Primer Premier 5.0 package (Premier Biosoft International), which was used for the first round of nested PCR amplification to enrich the amount of fungal DNA present in the sample. The species-specific primers were designed using the software Primer Premier 5.0 package according to the idiosyncratic ITS sequences of the *V. mali* var. *mali* isolates SXYL29 (JN575597), GSZL86 (JN412603), and NXLW92 (JN412604). The species-specific primers were preliminarily evaluated for their specificity using the Primer-Blast program in the GenBank.

Specificity of the primers. The specificity of the primers was evaluated using closely related *Valsa* spp. such as *V. malicola*, *V. sordida*, *V. persoonii*, and a *Valsa* sp. Also some distantly related

species isolated from infected apple tissues such as *Fusarium* spp., *Alternaria* spp., and *Penicillium digitatum* (Pers.) Sacc., and a *Puccinia striiformis* f. sp. *tritici* isolate representing a pathogen from a herbaceous plant, were also included for this purpose. Finally, some pathogens causing similar canker diseases on apple twigs were included as well (Table 1). In addition, five *V. mali* var. *pyri* isolates isolated from apple and five *V. mali* var. *pyri* isolates isolated from pear were also used to test the specificity of the primers.

The nested PCR was performed using a DNA thermal cycler PTC-200 (Bio-Rad) under the following conditions for the first run of PCR reactions (25.0 µl): 1× PCR buffer (75 mM Tris-HCl [pH 8.8], 20 mM [NH₄]₂SO₄, and 0.01% Tween 20), 2.5 mM MgCl₂, 0.1 mM dNTP mixture, 0.4 µM genus-specific primer *Valsa* forward (VF) and *Valsa* reverse (VR), 1.25 unit of Taq DNA polymerase (Fermentas), and approximately 10 ng of total DNA. The amplification reactions were carried out under the following conditions: 94°C for 4 min; followed by 40 cycles of 94°C for 30 s, 64.7°C for 30 s, and 72°C for 1 min; and ending with a final extension at 72°C for 10 min. The reactions lacking template DNA served as a negative control and the known *V. mali* var. *mali* species isolate \$XYC46 were used as a positive control (Table 1).

For the second round of amplification, the reaction mixtures (25.0 µl) contained 1× PCR buffer (75 mM Tris-HCl [pH 8.8], 20 mM [NH₄]₂SO₄, and 0.01% Tween 20), 2.5 mM MgCl₂, 0.1 mM dNTP mixture, 0.4 µM species-specific primer *V. mali* var. *mali* forward (VmF) and *V. mali* var. *mali* reverse (VmR), 1.25 unit of Taq DNA polymerase (Fermentas), and 1.0 µl of the first-run PCR amplification product as a template. The following PCR amplification conditions were employed: initial denaturation step of 4 min at 94°C; followed by 35 cycles of 30 s at 94°C, 45 s at 64.3°C, and 1 min at 72°C; and ending with a final extension of 10 min at 72°C. Finally, the PCR products were rapidly cooled to 4°C.

The PCR products were separated on 1.5% agarose gel stained with ethidium bromide in Tris-acetate buffer and photographed with a Molecular Imager gel imaging system (Bio-Rad).

Sensitivity of the assay. To test sensitivity of the nested PCR assay, DNA extracts from *V. mali* var. *mali* isolate 03-8-1 mycelium and apple tree bark tissues with or without visible symptoms were used as templates for the first run of PCR under the conditions described above. The DNA extracts from fungal mycelium and apple bark tissues showing visible symptoms were diluted to 400, 40, 4, and 1 ng/µl; 100, 10, and 1 pg/µl; and 100, 10, and 1 fg/µl for use as templates. The DNA extracts from apple bark tissues without visible symptoms were diluted to 300, 30, 3, and 1 ng/µl; 100, 10, and 1 pg/µl; and 100, 10, and 1 fg/µl. The second PCR amplification using species-specific primers VmF and VmR was conducted using the parameters as described above.

Nested PCR detection of *V. mali* var. *mali* on artificially inoculated apple twigs. One isolate of *V. mali* var. *mali* from Shaanxi province (03-8-1) was used to inoculate healthy symptomless, 2-year-old 'Fuji' apple twigs. The isolates were cultured on PDA for 3 days prior to inoculation. The twigs were washed in tap water and cut into short sections (about 45 cm long), dipped into 70% ethanol for 3 min, washed in sterile water twice, and air dried. In total, 10 twigs were used for inoculation. The cut twigs were sealed with hot paraffin wax at the morphological upper end, and the burn wounds were made with a nail head (8 mm in diameter) which was heated in the flame of alcohol lamp. There were three inoculated sites on each twig. A plug of agar with mycelium was affixed on the wound and sealed with Parafilm M (Parafilm). The twigs were planted in wet sand in 20-cm-diameter pots. The negative control consisted of a twig inoculated with a sterile plug of PDA. Following 20 days of growth in a greenhouse, the diseased tissue around the inoculation sites (approximately 1.5 cm long) was removed for DNA extraction, and the nested PCR assay was performed as described previously.

Table 1. Isolates of *Valsa mali* var. *mali*, *V. mali* var. *pyri*, and other fungal species used for determining the specificity of species-specific primers in the nested polymerase chain reaction amplification

Species	Isolate number	Host	Location	Year	GenBank number
<i>V. mali</i> var. <i>mali</i>	SXMX02	<i>Malus domestica</i> Borkh.	Meixian, Shaanxi	2003	JN412598
<i>V. mali</i> var. <i>mali</i>	03-3-1	<i>M. domestica</i>	Qianyan, Shaanxi	2003	JN418997
<i>V. mali</i> var. <i>mali</i>	03-8-1	<i>M. domestica</i>	Qianxian, Shaanxi	2003	JN412599
<i>V. mali</i> var. <i>mali</i>	HBBD10	<i>M. domestica</i>	Baoding, Hebei	2003	JN412606
<i>V. mali</i> var. <i>mali</i>	SXYL29	<i>M. domestica</i>	Yangling, Shaanxi	2003	JN575597
<i>V. mali</i> var. <i>mali</i>	SXFF43	<i>M. domestica</i>	Fufeng, Shaanxi	2003	JN412600
<i>V. mali</i> var. <i>mali</i>	\$XYC46	<i>M. domestica</i>	Yuncheng, Shanxi	2003	JN412601
<i>V. mali</i> var. <i>mali</i>	HNXX47	<i>M. domestica</i>	Xinxiang, Henan	2003	JN412607
<i>V. mali</i> var. <i>mali</i>	SXPC69	<i>M. domestica</i>	Pucheng, Shaanxi	2003	JN412602
<i>V. mali</i> var. <i>mali</i>	GSLZ86	<i>M. domestica</i>	Lanzhou, Gansu	2005	JN412603
<i>V. mali</i> var. <i>mali</i>	NXLW92	<i>M. domestica</i>	Lingwu, Ningxia	2005	JN412604
<i>V. mali</i> var. <i>mali</i>	SDQX118	<i>M. domestica</i>	Qixia, Shandong	2005	JN412605
<i>V. mali</i> var. <i>pyri</i>	03-14-1	<i>M. domestica</i>	Yangling, Shaanxi	2003	JN673554
<i>V. mali</i> var. <i>pyri</i>	SXYL126	<i>M. domestica</i>	Yangling, Shaanxi	2005	JN662365
<i>V. mali</i> var. <i>pyri</i>	SXYL134	<i>M. domestica</i>	Yangling, Shaanxi	2005	JN662366
<i>V. mali</i> var. <i>pyri</i>	SXYL135	<i>M. domestica</i>	Yangling, Shaanxi	2005	JN662367
<i>V. mali</i> var. <i>pyri</i>	GSZY113	<i>M. pumila</i>	Zhangye, Gansu	2005	GU174589
<i>V. mali</i> var. <i>pyri</i>	NXLW96	<i>Pyrus</i> spp.	Lingwu, Ningxia	2005	JN662368
<i>V. mali</i> var. <i>pyri</i>	SXBXp157	<i>Pyrus</i> spp.	Binxian, Shaanxi	2005	JN662369
<i>V. mali</i> var. <i>pyri</i>	GSZYpm160	<i>Pyrus</i> spp.	Zhangye, Gansu	2005	JN662370
<i>V. mali</i> var. <i>pyri</i>	NXLWp161	<i>Pyrus</i> spp.	Lingwu, Ningxia	2005	JN662371
<i>V. mali</i> var. <i>pyri</i>	SXWBp162	<i>Pyrus</i> spp.	Wubu, Shaanxi	2005	JN662372
<i>V. malicola</i>	03-1-2	<i>M. domestica</i>	Qishan, Shaanxi	2003	JN545839
<i>V. persoonii</i>	32-2w	<i>M. domestica</i>	Yangling, Shaanxi	2003	JN584644
<i>V. sordida</i>	YSFL	<i>Populus tomentosa</i>	Yangling, Shaanxi	2009	JN545841
<i>Valsa</i> sp.	LSFL	<i>Salix babylonica</i>	Yangling, Shaanxi	2009	JN575604
<i>Alternaria</i> sp.	LGB	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN575601
<i>Botryosphaeria dothidea</i>	PGLW	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN545840
<i>Cladosporium</i> sp.	PZM	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN575603
<i>Fusarium</i> sp.	LDJ	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN575600
<i>Penicillium digitatum</i>	QMPdi	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN575602
<i>Phoma glomerata</i>	JDMgl	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN575598
<i>Phoma</i> sp.	JDMsp	<i>M. domestica</i>	Yangling, Shaanxi	2009	JN575599
<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	TZ33	<i>Triticum aestivum</i>	Yangling, Shaanxi	2008	JN575605

Sequencing of nested PCR detection products. The DNA extractions from pure culture, symptomless bark tissues, naturally infected canker tissues, and artificially inoculated apple twig tissues were used as templates in the nested PCR, after which the PCR products were purified using an agarose Gel DNA Extraction Kit (Generay). The purified PCR products were directly sequenced

independently along both strands with species-specific primers VmF and VmR, and then the sequences were aligned using the DNAMAN 6.0 software package (Lynnon Biosoft).

Evaluation of two methods for detecting *V. mali* var. *mali* in apple twigs. All samples used in this experiment were derived either from symptomless tissues or infected tissues with visible

Fig. 1. Specificity of genus-specific and species-specific primers for *Valsa mali* var. *mali* in the **A**, first and **B**, second run of nested polymerase chain reaction, respectively, using DNA from *V. mali* var. *mali* isolates and other species. Lanes 1–10, *V. mali* var. *mali* isolates SXM02, 03-8-1, SXFF43, \$XYC46, SXPC69, GSLZ86, NXLW92, SDQX118, HBBD10, and HNXX47, respectively; lanes 11–20, *Cladosporium* sp., *Penicillium digitatum*, *Phoma glomerata*, *Phoma* sp., *Fusarium* sp., *Puccinia striiformis* f. sp. *tritici*, *Valsa* sp., *Alternaria* sp., *V. sordida*, and *Botryosphaeria dothidea*, respectively; lanes 21–23, DNA from *V. malicola* isolates 03-1-2, *V. mali* var. *mali* isolate \$XYC46, and *V. persoonii* isolate 32-2w, respectively; *V. mali* var. *mali* isolate \$XYC46 was used as positive control; CK, negative control (no DNA template); M, DL-2000 DNA ladder.

Fig. 2. Amplification products of the **A**, first and **B**, second run of nested polymerase chain reaction using the genomic DNA of *Valsa mali* var. *mali* and *V. mali* var. *pyri*, respectively. Lanes 1–10, *V. mali* var. *mali* isolates SXM02, 03-8-1, SXFF43, \$XYC46, SXPC69, GSLZ86, NXLW92, SDQX118, HBBD10, and HNXX47, respectively, isolated from apple; lanes 11–15, *V. mali* var. *pyri* isolates 03-14-1, SXYL126, SXYL134, SXYL135, and GSZY113, respectively, isolated from apple; lanes 16–20, *V. mali* var. *pyri* isolates NXLW96, SXBXp157, GSZYpm160, NXLWp161, and SXWBp162, respectively, isolated from pear; lanes 21 and 22, DNA of *V. malicola* isolate 03-1-2 and *V. persoonii* isolate 32-2w, respectively; CK, negative control (no DNA template); M, DL-2000 DNA ladder.

symptoms. The infected samples included twigs with canker symptoms showing visible pycnidia or soaked periderm tissue. The symptomless samples were the twigs showing no visible cankers. All samples were randomly collected from different apple orchards and washed with tap water. Then, a 1.5-cm² sample was cut in half. One half was used to isolate the fungal species present in pure culture while the other half was cut into small pieces and ground to a fine powder for DNA extraction. The DNA samples isolated were used as a template for the first round of nested PCR amplification.

Detection of *V. mali* var. *mali* from different apple tissues by nested PCR. DNA was extracted from different apple tissues as described above. The nested PCR assay using the genus-specific primers VF + VR and species-specific primers VmF + VmR was conducted as described above. The presence of *V. mali* var. *mali* in different tissues of apple trees was analyzed statistically using the SNK test (*Q* test) (SAS 8.0; SAS institute Inc.).

Results

Primer design. Using the ITS1 and ITS4 primers, we were able to detect an amplified DNA fragment of 560 bp from *V. mali* var. *mali*. This DNA fragment was subjected to sequence analysis. Based on the rDNA-ITS conserved sequence of the *Valsa* genus, a pair of genus-specific primers designated as VF (5'-GTT GCC TCG GCG CTG GCT-3') and VR (5'-GCG AGG GTT TTA CTA CTG C-3') was designed. Using this pair of primers in the nested PCR assay, a DNA fragment of approximately 400 bp was detected from *V. mali* var. *mali* in the first round of amplification (Figs. 1A and 2A). Based on the rDNA-ITS idiosyncratic sequence of *V. mali* var. *mali*, a pair of species-specific primers, VmF (5'-GAG TAC TCC CTC CCG CTC CG-3') and VmR (5'-TTA ATT AAG GGG CGG CCT CA-3'), was designed. Using this pair of primers in the second round

of amplification, a DNA fragment of 348 bp was specifically detected from *V. mali* var. *mali*.

Specificity of the assay. The specificity of this assay using the species-specific primers designed for detecting *V. mali* var. *mali* was evaluated by testing various isolates of *Valsa* and other fungal species which served as negative controls (Table 1). Using these primers (VmF and -R), the nested PCR was able to amplify a DNA fragment with an expected size of 348 bp when DNA from all *V. mali* var. *mali* isolates was used as a template but was unable to do so when DNA from isolates of other fungal species and *V. mali* var. *pyri* was used (Figs. 1B and 2B). Thus, this nested PCR assay could be applied specifically for the detection of *V. mali* var. *mali* present in apple trees.

Sensitivity of the assay. The sensitivity of the assay was tested by using the DNA extracted from a pure culture of *V. mali* var. *mali* after a serial DNA dilution from 400 ng/μl to 1 fg/μl. The results of this study revealed that this assay was able to detect a 348-bp DNA fragment when the tested DNA concentrations were in the range of 100 fg/μl to 400 ng/μl. Thus, when the conserved primers (VF and VR) were used in the first round of amplification and species-specific primers (VmF and VmR) were used in the second round of amplification in the nested PCR assay, *V. mali* var. *mali* could be detected in mycelial samples at DNA concentrations as low as 100 fg/μl (Fig. 3A). When DNA extracts from apple bark tissues with visible symptoms were used as a template in the nested PCR, the same result was obtained (Fig. 3B). However, when DNA extracts from symptomless apple bark tissue were used, the lowest detectable concentration was 10 pg/μl (Fig. 3C). These data show that the assay is highly sensitive for detection of *V. mali* var. *mali* in different types of samples.

Nested PCR detection in different apple samples and sequencing of PCR products. The nested PCR was used to detect *V. mali* var. *mali* in artificially inoculated apple twigs, pure cultures, naturally infected canker tissues, and symptomless bark tissues, after which the PCR products were sequenced. The canker symptoms appeared on apple twigs 20 days after artificial inoculation. There were no obvious canker symptoms in the controls inoculated with PDA plugs. The DNA was extracted from the infected tissues, and an expected PCR product with a size of 348 bp was observed when the DNA isolated from the infected tissues was used as a template in the nested PCR assay. Similarly, when DNA isolated from pure culture, symptomless bark tissues, and naturally infected canker tissues was used as a template, a PCR product with the same size was observed (Fig. 4). Sequencing results showed that the detected PCR products from these four kinds of samples had the same sequence as the PCR detection products from mycelium (Fig. 5). Therefore, they were considered to be the same species.

Validation of the assay by fungal isolation. To validate the nested PCR assay results, the conventional fungal isolation method was employed. In all, 40 naturally infected samples with symptoms and 34 samples without symptoms were tested. A positive PCR signal (i.e., a 348-bp PCR product) was observed in all tested natu-

Fig. 3. Sensitivity of nested polymerase chain reaction with species-specific primers VmF and VmR for detection of *Valsa mali* var. *mali*. **A**, DNA extracted from a pure culture of *V. mali* var. *mali* isolate 03-8-1 isolated from apple in Shaanxi province. Lanes 1–10, DNA at 400 ng, 40 ng, 4 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg/μl, respectively. **B**, DNA extracted from apple bark tissue with visible symptoms. Lanes 1–10, The same concentrations as **A**. **C**, DNA extracted from symptomless apple bark tissue. Lanes 1–10, DNA at 300 ng, 30 ng, 3 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg/μl, respectively. CK, negative control (no DNA template); M, DL-2000 DNA ladder.

Fig. 4. Detection of *Valsa mali* var. *mali* in naturally infected canker tissue, artificially inoculated twig tissue, and symptomless bark tissue by the nested polymerase chain reaction with species-specific primers. Lanes 1–3, DNA samples from naturally infected canker tissue; lanes 4–6, DNA samples from canker tissue on artificially inoculated twigs; lanes 7–9, DNA samples from symptomless bark tissue; lanes 10–12, DNA of *V. mali* var. *mali* isolate 03-3-1, *V. persoonii* isolate 32-2w, and *V. malicola* isolate 03-1-2, respectively, from pure cultures; *V. mali* var. *mali* isolate 03-3-1 served as positive control; M, DS-2000 DNA ladder; CK, negative control (no DNA template).

rally infected samples. By the fungal isolation method, the pathogen was also detected in these samples. The detection rate of the nested PCR and fungal isolation for naturally infected samples was 100% in both cases. Among the 34 samples without obvious symptoms, 22 samples yielded a positive PCR signal while the remaining 12 samples did not. The detection rate of the nested PCR for symptomless samples was 64.7% (22/34). Subsequently, we were able to isolate *V. mali* var. *mali* from seven of the symptomless samples. Meanwhile, the presence of *V. mali* var. *mali* in these seven samples was also confirmed by the nested PCR. Moreover, the seven samples were included in the 22 samples that yielded a positive PCR signal. There were 15 samples in which the presence of the pathogen was detected by the nested PCR assay but failed by fungal isolation. Therefore, the detection rate of fungal isolation for symptomless samples was 20.6% (7/34), which was much lower than the detection rate of 64.7% by nested PCR. There were no samples from which the fungi could be isolated but failed to be detected by the nested PCR. These data indicate that the nested PCR is more sensitive and reliable than fungal isolation.

Comparison of *V. mali* var. *mali* infection in symptomless apple tissues in orchards with different diseased tree incidence. In this study, the presence of *V. mali* var. *mali* in symptomless apple tissues (terminal buds, internodes, and bud scale scars) was evaluated by nested PCR. In three orchards where the diseased tree incidence was 80% in each, 179 symptomless samples in all from different tissues were collected and analyzed by the PCR assay, and *V. mali* var. *mali* was detected in 150 of the samples. Thus, the incidence of *V. mali* var. *mali* in symptomless apple tissues in these orchards was 83%, similar to the diseased tree incidence. Yet, in another three orchards where the diseased tree incidence was 28, 40, and 36%, respectively (on average, 34.7%), 177 symptomless samples in total were collected and analyzed by the PCR assay, and the presence of *V. mali* var. *mali* was detected in 97(55%) of the samples. The orchards were divided into two types according to the

diseased tree incidence: type I was composed of orchards with a high diseased tree incidence (80%) and type II consisted of orchards with a moderate diseased tree incidence (<40%). The *Q* test result showed that the incidence of *V. mali* var. *mali* in various plant tissues in high-disease-incidence orchards was significantly different from those with moderate disease incidence ($F = 10.61$, $P = 0.0069$). Furthermore, our results showed that *V. mali* var. *mali* was present in all tested symptomless tissues on apple trees. The statistical analysis also indicated that the incidence of *V. mali* var. *mali* in different tissues was significantly different ($F = 7.11$, $P = 0.0092$). *V. mali* var. *mali* was detected in 89% of tested terminal bud samples on average. In internodes, the incidence was 71% on average, and the detection rate was 48% in bud scale scars (Table 2).

Discussion

In this study, a PCR-based assay for detecting the pathogen causing Valsa canker (namely, *V. mali* var. *mali*) in apple trees was developed. This PCR assay could be used for rapid and accurate detection of the causal agent *V. mali* var. *mali* in different tissues with or without visible canker symptoms. Moreover, compared with the traditional fungal isolation method, the nested PCR assay is more sensitive in detecting *V. mali* var. *mali* from symptomless tissues. Therefore, the *V. mali* var. *mali* incidence in orchards or in different tissues can be monitored much earlier by the PCR assay, at an early stage of infection before symptoms appear. It was very critical for effectively controlling disease at this early stage of infection by protective fungicides. Otherwise, it is very difficult to control disease by inhibiting pathogen development in the bark tissues because the mycelia have been invading in the phloem and even deeper tissues. Our assay could provide an exact prediction of the disease in the orchard to help growers take control measures as early as possible. In addition, it is easy for the fungicides to enter the outer layers of the bark to inhibit mycelia growth. Moreover, by

AIT	GAGTACTCCCTCCGCTCCGGGAGGGGGCCGCTCTGGTCGTAAGAACCAGGGGAGGACAGCAGGCCC GCGCGGGCC	80
NIT	GAGTACTCCCTCCGCTCCGGGAGGGGGCCGCTCTGGTCGTAAGAACCAGGGGAGGACAGCAGGCCC GCGCGGGCC	80
SBT	GAGTACTCCCTCCGCTCCGGGAGGGGGCCGCTCTGGTCGTAAGAACCAGGGGAGGACAGCAGGCCC GCGCGGGCC	80
MYT	GAGTACTCCCTCCGCTCCGGGAGGGGGCCGCTCTGGTCGTAAGAACCAGGGGAGGACAGCAGGCCC GCGCGGGCC	80
Con	gagtactccctcccgctccgggagggggcccgctctggtcgtaagaaccaggggaggacagcagggcccgcgcgcc	
AIT	CAATTAACCTCTGTATTTACCGAGTAAATCTGAGTAAGCTTCTAAATGAATCAAACTTTCAACAACGGATCTCTTGGT	160
NIT	CAATTAACCTCTGTATTTACCGAGTAAATCTGAGTAAGCTTCTAAATGAATCAAACTTTCAACAACGGATCTCTTGGT	160
SBT	CAATTAACCTCTGTATTTACCGAGTAAATCTGAGTAAGCTTCTAAATGAATCAAACTTTCAACAACGGATCTCTTGGT	160
MYT	CAATTAACCTCTGTATTTACCGAGTAAATCTGAGTAAGCTTCTAAATGAATCAAACTTTCAACAACGGATCTCTTGGT	160
Con	caattaactcttgtatttacogagtaaaatctgagtaagcttctaataatgaatcaaaactttcaacaacggatctcttgtt	
AIT	TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTCAGTGAATCATCGAATCTTTGAAC	240
NIT	TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTCAGTGAATCATCGAATCTTTGAAC	240
SBT	TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTCAGTGAATCATCGAATCTTTGAAC	240
MYT	TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTCAGTGAATCATCGAATCTTTGAAC	240
Con	tctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaattgcagaattcagtgaaatcatcgaatctttgaac	
AIT	GCACATTGCGCCCTCTGGTATTCCAGAGGGCATGCCGTGTCGAGCGTCATTTCACCCCTCAAGCCCTGCTTGGTGTGGG	320
NIT	GCACATTGCGCCCTCTGGTATTCCAGAGGGCATGCCGTGTCGAGCGTCATTTCACCCCTCAAGCCCTGCTTGGTGTGGG	320
SBT	GCACATTGCGCCCTCTGGTATTCCAGAGGGCATGCCGTGTCGAGCGTCATTTCACCCCTCAAGCCCTGCTTGGTGTGGG	320
MYT	GCACATTGCGCCCTCTGGTATTCCAGAGGGCATGCCGTGTCGAGCGTCATTTCACCCCTCAAGCCCTGCTTGGTGTGGG	320
Con	gcacattgcgccctctggtattccagagggcatgacctgttcgagcgctcatttcaacctcaagccctgcttgggtgtggg	
AIT	GCATTACCTGAGGCGCCCTTAATTAA	348
NIT	GCATTACCTGAGGCGCCCTTAATTAA	348
SBT	GCATTACCTGAGGCGCCCTTAATTAA	348
MYT	GCATTACCTGAGGCGCCCTTAATTAA	348
Con	gcattacctgagggcgcccttaattaa	

Fig. 5. Multiple sequence alignment of the nested polymerase chain reaction products from naturally infected canker tissue (NIT), artificially inoculated twigs tissue (AIT), symptomless bark tissue (SBT), and fungal mycelium (MYT) with species-specific primers VmF and VmR. Con, consensus sequence.

the detection of *V. mali* var. *mali* incidence in different tissues, the places where the pathogen survived can be found, which paves the way for much better understanding of the rules of disease development.

To develop a reliable and sensitive PCR assay, it was critically important to obtain genomic DNA samples of high quality for use as template for amplification. There are many factors that may hinder the isolation of high-quality fungal DNA from plant tissues suitable for PCR, such as sampling sites on twigs, sample processing, and presence of substances in plant tissue interfering with PCR (9). In our DNA extraction procedure, we added PVP and β -mercaptoethanol to prevent DNA degradation and also a larger amount of sodium acetate to facilitate DNA precipitation. We believe that these measures might have contributed to the isolation of high-quality genomic DNA samples for the assay. For efficient amplification, we believe that using sufficiently diluted DNA samples is critical. However, this could reduce sensitivity. In this study, we used the original DNA preparations without further dilution and obtained a strong and expected DNA fragment by the nested PCR, indicating that the assay developed in this study is sensitive and reproducible.

A reliable PCR assay also depends on the design and use of the primers specific to the intended target pathogens (8). In this study, we designed the species-specific primers according to the idiosyncratic ITS sequences of three *V. mali* var. *mali* isolates from different regions. Although variation was discovered in the ITS region of *V. mali* isolates, two varieties, *V. mali* var. *mali* and *V. mali* var. *pyri*, were recognized by seven nucleotide substitution sites in the ITS sequences. *V. mali* var. *mali* was more aggression than *V. mali* var. *pyri* on apple (18). Additionally, we found that *V. mali* var. *mali* was dominant, comprising 99.2% (729/735) of the isolates obtained from apple trees. These were also the reasons why we designed primers to detect *V. mali* var. *mali* rather than *V. mali* var. *pyri*. When we used these primers to test closely related fungal species, the primer pair VmF and -R amplified a DNA fragment with a size of 348 bp from *V. mali* var. *mali* isolates but failed to yield any obvious fragments from other fungal species tested. Furthermore, when DNA extracts from *V. mali* var. *mali* and *V. mali* var. *pyri* were used as templates in the nested PCR assay, the expected 348-bp DNA fragment was generated only in *V. mali* var. *mali* isolates. Thus, these results demonstrate that the primers VmF and -R used in this study are specific to the target pathogen. Whereas the one-step PCR can detect DNA concentrations as low as 40 ng/ μ l, the nested PCR assay can detect 100 fg/ μ l when DNA extracts from mycelium and symptomatic apple bark are used as templates. When DNA extracts from symptomless apple bark tissue were used as templates, the lowest detectable limit was DNA at 10 pg/ μ l; however, one-step regular PCR cannot detect pathogen in these samples at all (Fig. 6). Thus, the nested-PCR assay can be used to detect a latent pathogen in extremely low quantities within apple tree bark.

Subsequently, in our assay validation study, all the samples from which the pathogens were successfully isolated yielded a positive PCR signal, thereby confirming that the nested PCR assay is a sensitive and reliable detection method. In some cases, where fungi were not isolated from the samples but were detected by the nested PCR assay, this may be due to the lower sensitivity of the conventional isolation method. On the other hand, it is possible that the DNA extracted from dead *V. mali* var. *mali* cells still can be amplified using the nested PCR assay. Isolation is generally less efficient, because small hyphae may not survive the isolation process or are just not present in the small sections plated. These might have explained why, in some cases, the pathogen could be detected by the nested PCR assay but subsequently failed to be isolated by

Fig. 6. Sensitivity of one-step polymerase chain reaction with species-specific primers VmF and VmR for detection of *Valsa mali* var. *mali*. A, DNA extracted from a pure culture of *V. mali* var. *mali* isolate 03-8-1 isolated from apple in Shaanxi province. Lanes 1–10, DNA at 400 ng, 40 ng, 4 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg/ μ l, respectively. B, DNA extracted from apple bark tissue with visible symptoms. Lanes 1–10, The same concentrations as A. C, DNA extracted from symptomless apple bark tissue. Lanes 1–10, DNA at 300 ng, 30 ng, 3 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg/ μ l, respectively; CK, negative control (no DNA template); M, DL-2000 DNA ladder.

Table 2. Nested polymerase chain reaction (PCR) detection of *Valsa mali* var. *mali* incidence in different tissues of apple trees obtained from the orchards with different diseased tree incidence in Yangling, Shaanxi province in 2011

Orchard type, number	Diseased tree incidence (%) ^y	Incidence in tissue (%) ^x		
		Terminal bud ^z	Internode	Bud scale scar
I				
1	80	100	100	40
2	80	100	100	85
3	80	80	90	60
II				
4	28	75	60	5
5	40	100	60	55
6	36	80	15	41
Average incidence	...	89 a	71 ab	48 b

^x Lowercase letters represent horizontal comparison: shared letters indicate no significant difference at $P = 0.05$ using the SNK test (Q test).

^y Incidence of diseased trees in each orchard was calculated by evaluation of 25 trees.

^z In each orchard, five trees with typical symptoms were randomly selected and four 1-year-old symptomless twigs in four directions were cut from each tree; thus, 20 samples were tested by the nested PCR assay.

the isolation methods. Admittedly, because of the specificity of species-specific primers in the nested PCR, we are not sure, by the nested PCR, whether *V. mali* var. *pyri* exist in the naturally infected samples and some symptomless samples from which the expected 348-bp DNA fragment was not amplified. However, the pathogen was not obtained by the fungal isolation method either and, therefore, the test appears reliable.

According to the detection results, we now know that *V. mali* var. *mali* incidence in symptomless tissues was over 50% (55%) in the orchards in Shaanxi Province. The results suggest that *V. mali* var. *mali* can invade and colonize many apple tree tissues. We infer that this pathogenic fungus could cause latent infection for a long time and survive in any weak and dead tissues on the apple trees. The similar pathogen *Cytospora chrysosperma* (Pers.) Fr. also might cause latent infection, as suggested by Christensen et al. (5). On the basis of this finding, we advised the growers to change their disease control strategy from treatments after symptoms appear to prevention measures prior to the appearance of visible cankers. Our results suggest that the growers could control disease by taking different measures to reduce inoculum concentrations and latent infections; for example, spraying fungicides over the whole trees and on areas on the trunks or scaffold limbs where cankers have been surgically removed, and pruning the weak, dead twigs. Moreover, the infected twigs and bark tissues with symptoms were promptly removed and deeply buried or burned rather than piled up in the orchards. A proper utilization of fertilization and irrigation was necessary to improve the vigor of trees to aid in reducing the infection. The results of this study have improved the understanding of inoculum sources of *V. mali* var. *mali* in apple orchards in western China and suggest avenues for improved management of the disease.

However, the nested PCR assay was based on the fungal genomic DNA which might be extracted from living and dead fungal cells. Therefore, for better understanding and more accurate detection of the fungus infection, real-time reverse-transcription PCR should have been used to confirm viability of fungal cells.

Meanwhile, due to the fact that these studies were conducted using samples collected during the harvest season, it is possible that the distribution of this pathogen in different tissues may vary in different seasons. For example, the *Cytospora* pathogens on many trees could attack the hosts as facultative wound parasites when the vigor of the hosts had declined (2). Many factors can cause poor vigor of the hosts, such as drought, freezing, winter injury, or severe pruning. The vigor of the apple trees might vary in different seasons. Therefore, it is important to conduct these types of studies using samples collected during different growing periods in order to have a better understanding of the epidemic development of Valsa canker in apple trees.

In summary, the nested PCR assay developed in this study is highly sensitive and specific for detecting the presence of *V. mali* var. *mali* in apple trees as compared with the traditional culture isolation method. This nested PCR assay is suitable for detecting the fungal pathogen present in tissues with and without symptoms. This method could also be used to predict and monitor the presence of this pathogen at early stages of disease development, which is critical to gain insight into the epidemiology of the disease, thereby aiding the development of effective strategies for controlling the disease.

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