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Detection of *Sclerotinia sclerotiorum* Using a Monomeric and Dimeric Single-Chain Fragment Variable (scFv) Antibody

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Sclerotinia sclerotiorum (Lib.) de Bary is a phytopathogenic fungus capable of causing significant yield losses in numerous crops, including canola, in which the fungus causes sclerotinia stem rot. Immunological detection methods to rapidly determine the presence of *S. sclerotiorum* on plants may provide growers with a viable diagnostic tool to aid with fungicide use decisions. This paper discusses the generation of a monomeric and dimeric single-chain, variable fragment (scFv) antibody with affinity for *S. sclerotiorum* using phage display technology. The bacterially expressed and purified scFv is shown to bind *S. sclerotiorum* with some cross-reactivity with the closely related phytopathogen *Botrytis cinerea* (Pers.:Fr.). The dimeric scFv displayed improved binding to the fungus as compared to the monomer and could detect the presence of mycelia in inoculated canola petals. To the authors' knowledge, this is the first report of a scFv dimer with affinity for *S. sclerotiorum* that has the potential for use in the development of a new diagnostic test.

KEYWORDS: Canola; phage display; phytopathogen; scFv; *Sclerotinia sclerotiorum*

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a well-known necrotrophic phytopathogenic fungus with over 400 different potential plant hosts (*1, 2*). The wide range of host plants includes numerous agriculturally important crops such as canola, in which the fungus is capable of causing the disease sclerotinia stem rot (*3*). Canola is grown in Europe and Asia as well as in North America, where, in Canada, the harvested acreage exceeded 14.6 million acres, leading to over 8.7 million tonnes of canola produced in 2007 (*4*). If stem rot inoculum is present in adequate quantities and environmental conditions are favorable for infection, yield loss per plant can be as high as 50% (*5*). Risk assessment tools or disease management strategies that can be used to minimize the deleterious effects of *S. sclerotiorum* include the use of disease-forecasting protocols such as a petal test kit and a checklist or survey, appropriate seeding to prevent high crop density, and fungicide application (*6*−*11*). Currently, there are only canola cultivars that are partially resistant to *S. sclerotiorum* infection, and there are few genetic sources of resistance for breeders, which highlights the importance of effective disease forecasting and management procedures (*12, 13*). In addition, the diverse range of plants that can act as hosts as well as the longevity of sclerotia can limit the effectiveness of crop rotations (*14*). Furthermore, novel tests to detect the presence of *S. sclerotiorum* in a particular field or during a particular growing season may supplement currently used disease-forecasting systems and provide canola growers with another tool to assess stem rot risk and thus determine the need for fungicide to limit the damage caused by this fungus.

Previous attempts at using antibodies to detect *S. sclerotiorum* were based on polyclonal antibodies (*15, 16*). Antibody production requires the immunization of animals followed by collection of serum containing the antibodies generated by the immune systems of the animals. A potential drawback of polyclonal antibody production is the limited amount of serum that can be collected from individual animals, which necessitates continual immunization. Furthermore, the polyclonal antibodies in the collected serum are diverse in terms of which epitopes are recognized and, hence, may have a broader cross-reactivity. An alternative involves the production and use of monoclonal antibodies using hybridoma technology (*17*). Unlike polyclonal antibody production, monoclonal antibodies can be produced so that each clone will produce antibodies that can bind to a specific epitope of a specific antigen. The nature of monoclonal antibody production also theoretically enables the continual production of specific antibodies provided that the hybridoma cells are properly maintained. Alternatively, recombinant antibody technology can be utilized to generate single-chain variable

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fragment (scFv) antibody fragments, which are smaller than whole immunoglobulins because they consist of only the variable heavy (V_H) and variable light (V_L) chains of a whole antibody linked by a short polypeptide linker (*18*). These antibodies retain the antigen-binding capacity of the whole antibodies from which they were derived and can be produced in relatively large amounts with bacterial expression systems (*19*−*21*).

The objectives of the current study include (1) the generation of scFv antibody fragments with affinity for the phytopathogenic fungus *S. sclerotiorum* using phage display technology, (2) the histology-based confirmation of scFv binding to *S. sclerotiorum* mycelia, (3) the determination of the specificity of the scFv for *S. sclerotiorum*, (4) the generation of scFv dimer and comparison of the dimer and monomer binding to *S. sclerotiorum* mycelia, and (5) the assessment of the potential utility of the scFv dimer to detect *S. sclerotiorum* on artificially infested canola petals. In this paper, we describe the isolation of a scFv antibody that recognizes and binds to *S. sclerotiorum* mycelia using phage display technology, and we discuss its specificity and binding to mycelia, the effects of multimerization, and its use to detect *S. sclerotiorum* on infected canola petals.

MATERIALS AND METHODS

Immunization of Mice. The stock of *S. sclerotiorum* used was isolated from infected canola and stored in liquid nitrogen as a pure glycerol stock. After culturing of the stock fungus on PDA plates and the development of sclerotia, individual sclerotia were sliced in half using sterile blades, and the sliced sclerotia were used to inoculate fresh PDA plates. The whole fungal mycelial antigen sample was prepared by growing *S. sclerotiorum* mycelia from PDA agar plugs in sterile liquid media (50 mM NH_4Cl, 7.3 mM KH_2PO_4, 4.2 mM $MgSO_4$, 6.7 mM KCl, 66 μM $FeSO_4$) supplemented with 1% (w/v) pectin for a period of 5 days with agitation at room temperature (100 rpm on a platform shaker), after which mycelia were collected and washed with phosphate-buffered saline (PBS) to eliminate any residual secreted proteins. Fungal growth and preparation were performed aseptically to avoid contamination.

All animal immunizations were approved by the Animal Care and Use Committee for Biosciences (University of Alberta) and were performed in accordance with Canadian Council on Animal Care guidelines by the Biological Sciences Animal Services unit (University of Alberta). Prior to the priming injection, tail bleeds were performed on 4−5-week-old BALB/c mice to collect preimmunization serum samples to use as controls in subsequent antibody titer assays. The mice were primed once with a maximum of 200 μL/mouse of Freund's complete adjuvant (FCA) from Difco (Franklin Lakes, NJ) containing 20 μg of *S. sclerotiorum* mycelia such that a maximum of 100 μL (10 μg of mycelia) was given per subcutaneous site. The frequency of boost immunizations was approximately 3 weeks apart over an 8−16 week period with a maximum of five boost injections per mouse. Each boost injection consisted of a maximum of 200 μL of Freund's incomplete adjuvant (FIA; Difco) containing 20 μg of mycelia such that a maximum of 100 μL (10 μg of mycelia) was given per subcutaneous site. The immune responses of the immunized mice were periodically tested using antibody titer assays, and once satisfactory antibody titers had been obtained, the mice were euthanized. Four days before euthanization, mycelia of *S. sclerotiorum* (20 μg) in 50 μL of sterile PBS at pH 7 were injected into the tail veins. The mice were euthanized by cervical fracture or with carbon dioxide, and the spleens were removed using aseptic technique and immediately placed in RNAlater solution from Ambion (Austin, TX) to stabilize the RNA.

Phage Display scFv Antibody Library Preparation. Total RNA and mRNA were isolated from the extracted mouse spleens with the RNeasy Mini Kit from Qiagen (Mississauga, ON, Canada) and the mRNA Purification kit from GE Healthcare (Piscataway, NJ), respectively and the corresponding cDNA was synthesized using the First Strand cDNA Synthesis Kit (GE Healthcare). The V_H and V_L antibody fragments were amplified by Polymerase Chain Reaction (PCR) using the primers listed in **Table 1** for final assembly in the orientation $V_L-(G_4S)_4-V_H$ (*18*). After an initial DNA denaturation step at 92 °C for 3 min, 7 cycles of 60 s at 92 °C, 30 s at 63 °C, 50 s at 58 °C, 60 s at 72 °C, and 30 cycles of 60 s at 92 °C, 30 s at 63 °C, 60 s at 72 °C were performed. The PCR was completed with a final incubation of 7 min at 72 °C. Splicing by overlap extension with the "scfor" and "scback" primers (**Table 1**) to link and amplify the V_L and V_H domains was performed using the same PCR cycle. Following gel purification of the antibody fragments, the scFv genes were digested with the restriction enzyme *Sfi*I from New England Biolabs (Ipswich, MA) and inserted into *Sfi*I-digested pAK100 vector, which was kindly provided by Dr. Andreas Pluckthun (University of Zurich). Competent TG1 *Escherichia coli* cells (500 μL) were transformed with the scFv antibody library and then plated onto 2× YT agar plates supplemented with 1% (w/v) glucose and 30 μg/mL chloramphenicol (2× YT-GC). The plates were incubated at 37 °C overnight to allow growth of transformed cells.

Table 1. Sequences of Primers Used for PCR Amplification of V_L and V_H Domains[a]

Heavy Chain Forward Primers
HF1: GGAATTCGGCCCCCGAGGCCGAGGAAACGGTGACCGTGGT
HF2: GGAATTCGGCCCCCGAGGCCGAGGAGACTGTGAGAGTGGT
HF3: GGAATTCGGCCCCCGAGGCCGCAGAGACAGTGACCAGAGT
HF4: GGAATTCGGCCCCCGAGGCCGAGGAGACGGTGACTGAGGT

Heavy Chain Reverse Primers
HB1: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAKGTRMAGCTTCAGGAGTC
HB2: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTBCAGCTGCAGCAGTC
HB3′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCCAGGTGCAGCTGAAGSARTC
HB4: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTCCARCTGCAACARTC
HB5: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCCAGGTYCAGCTBCAGCARTC
HB6′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCCAGGTYCARCTGCAGCARTC
HB7′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCCAGGTCCACGTGAAGCARTC
HB8′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTGAASSTGGTGGARTC
HB9′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAVGTGAWGSTGGTGGAGTC
HB10′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTGCAGSTGGTGGARTC
HB11′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAKGTGCAMCTGGTGGARTC
HB12: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTGAAGCTGATGGARTC
HB13′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTGCARCTTGTTGARTC
HB14′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGARGTRAAGCTTCTCGARTC
HB15′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAAGTGAARSTTGAGGARTC
HB16′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCCAGGTTACTCTRAAASARTC
HB17: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCCAGGTCCAACTVCAGCARCC
HB18′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGATGTGAACTTGGAASARTC
HB19′: GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTGAAGGTCATCGARTC

Light Chain Forward Primers
LF1′: GGAGCCGCCGCCGCC(AGAACCACCACCACC)$_2$ACGTTTKATTTCCAGCTTGG
LF4: GGAGCCGCCGCCGCC(AGAACCACCACCACC)$_2$ACGTTTTATTTCCAACTTTG
LF5: GGAGCCGCCGCCGCC(AGAACCACCACCACC)$_2$ACGTTTCAGCTCCAGCTTGG
LFλ: GGAGCCGCCGCCGCC(AGAACCACCACCACC)$_2$ACCTAGGACAGTCAGTTTGG

Light Chain Reverse Primers
LB1: GCCATGGCGGACTACAAAGAYATCCAGCTGACTCAGCC
LB2: GCCATGGCGGACTACAAAGAYATTGTTCTCWCCCAGTC
LB3: GCCATGGCGGACTACAAAGAYATTGTGMTMACTCAGTC
LB4: GCCATGGCGGACTACAAAGAYATTGTGYTRACACAGTC
LB5: GCCATGGCGGACTACAAAGAYATTGTRATGACMCAGTC
LB6: GCCATGGCGGACTACAAAGAYATTMAGATRAMCCAGTC
LB7: GCCATGGCGGACTACAAAGAYATTCAGATGAYDCAGTC
LB8: GCCATGGCGGACTACAAAGAYATYCAGATGACACAGAC
LB9: GCCATGGCGGACTACAAAGAYATTGTTCTCAWCCAGTC
LB10: GCCATGGCGGACTACAAAGAYATTGWGCTSACCCAATC
LB11: GCCATGGCGGACTACAAAGAYATTSTRATGACCCARTC
LB12: GCCATGGCGGACTACAAAGAYRTTKTGATGACCCARAC
LB13: GCCATGGCGGACTACAAAGAYATTGTGATGACBCAGKC
LB14: GCCATGGCGGACTACAAAGAYATTGTGATAACYCAGGA
LB15: GCCATGGCGGACTACAAAGAYATTGTGATGACCCAGWT
LB16: GCCATGGCGGACTACAAAGAYATTGTGATGACACAACC
LB17: GCCATGGCGGACTACAAAGAYATTTTGCTGACTCAGTC
LBλ: GCCATGGCGGACTACAAAGATGCTGTTGTGACTCAGGAATC

scforward Primer
GGAATTCGGCCCCCGAG

scback Primer
TTACTCGCGGCCCAGCCGGCCATGGCGGACTACAAAG

[a] The listed sequences are written in the 5′ to 3′ direction and are provided using the IUPAC nomenclature of mixed bases (R = A or G, M = A or C, Y = C or T, K = G or T, S = C or G, W = A or T, H = A or C or T, B = C or G or T, V = A or C or G, and D = A or G or T).

The construction of the phage display scFv library, panning, and phage rescue were performed essentially as described in refs *18, 22*, and *23* with minor modifications.

Phage Rescue and Panning for Isolation of Strong Binders. Transformants were resuspended in 5 mL of 2× YT-GC, which was then used to inoculate 5 mL of fresh 2× YT-GC, and the culture was incubated at 37 °C at 250 rpm until the OD_{600nm} reached approximately 0.5. Five milliliters of fresh 2× YT-GC medium containing 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 5×10^9 cfu VCSM13 helper phage from Stratagene (La Jolla, CA) was added to the culture, which was incubated overnight at room temperature with agitation (250 rpm). The culture was centrifuged (1000*g*, 20 min, 22 °C), and the supernatant containing the scFv-displaying phage was collected. The supernatant was mixed with 2 mL of sterile PEG/NaCl solution [20% (w/v) PEG 8000 and 14.6% (w/v) NaCl], vortexed briefly, and incubated on ice for 1 h to precipitate the scFv-displaying phage. The precipitated phage was collected by centrifugation (10000*g*, 20 min, 4 °C) followed by removal of the supernatant. The phage pellet was resuspended in 1 mL of PBS, and the phage titer was determined using serial dilutions in 100 μL of PBS (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12}). To each of the dilutions, 900 μL of TG1 *E. coli* in 2× YT ($OD_{600nm} \sim 0.4$) was added, and the samples were incubated at 37 °C for 30 min. One hundred microliters of each sample was spread evenly on the surface of 2× YT-GC agar plates, which were incubated overnight at 30 °C. The colonies on the plates were counted to calculate the phage titer. The phage suspension was diluted with an equal volume of PBS−BSA for a final concentration of 1% BSA (w/v) and then used to pan for the isolation of *S. sclerotiorum* mycelia binders as described below.

Wells of an 8-well Flat Bottom ImmunoModule from Nunc (Rochester, NY) were coated with 100 μL/well of 10 μg/mL mycelial homogenates in PBS. PBS (100 μL/well) alone was added to other wells to serve as uncoated controls. The wells were incubated for 2 days at 30 °C and then washed three times with PBS. Blocking solution [2% (w/v) BSA in PBS] was added (200 μL/well), and wells were incubated with agitation (∼100 rpm) for 1 h at room temperature. After three washings of the wells with PBS, the phage suspension was added to the wells (10^{10} phage/well), which were incubated at room temperature for 2 h with agitation (∼100 rpm). The wells were washed 20 times with sterile PBS containing 0.05% (v/v) Tween-20 followed by 20 washings with sterile PBS. The bound phage was eluted by the addition of sodium acetate buffer (100 μL/well; 0.1 M acetic acid, 0.15 M NaCl, pH 2.8) and incubation for 8 min followed by neutralization with 2 M Tris buffer, pH 9.5 (12 μL/well). The neutralized phage solutions were used immediately to infect 5 mL of log-phase TG1 *E. coli* cells ($OD_{600nm} \sim 0.5$) for 30 min at 37 °C followed by an additional 30 min at room temperature. The TG1 cells were centrifuged (3444*g*, 10 min, 4 °C), 4 mL of the supernatant was removed, and the pellets were resuspended in the remaining liquid. The cells were spread evenly on 2× YT-GC agar plates (200 μL/plate), which were incubated at 28 °C overnight. The colonies were either used in a phage ELISA (see below) to identify antigen binders or pooled and used in another round of panning. At least two rounds of panning were required to isolate strong antigen binders.

Phage ELISA. After a round of panning, randomly selected colonies were used to inoculate separate 5 mL of 2× YT-GC medium in sterile polypropylene tubes. The tubes were incubated overnight at 37 °C with agitation (250 rpm on platform shaker). Fresh 2× YT-GC medium (5 mL) was inoculated with 20 μL of the overnight culture and incubated with agitation at 250 rpm at 37 °C until an OD_{600nm} of ∼0.5 was reached. Fresh 2× YT-GC medium (5 mL) supplemented with 1 mM IPTG and 5×10^9 cfu VCSM13 helper phage was added to each culture, which was then incubated overnight at room temperature with agitation (250 rpm). The cultures were centrifuged (1000*g*, 20 min, 4 °C), and the supernatants were added to separate 2 mL of sterile PEG/NaCl to precipitate the phage. After vortexing, the samples were incubated on ice for 1 h and then centrifuged at 10000*g* for 20 min at 4 °C. The supernatants were discarded, and the pellets were resuspended in 400 μL of PBS. The phage titer was determined, and the phage suspension was diluted in PBS−BSA as described above. Eight-well Flat Bottom ImmunoModule wells were coated, incubated, blocked, and washed with PBS as described above. Phage suspensions were added to the wells (10^{10} phage/well), which were incubated at room temperature for 2 h with agitation (∼100 rpm). The wells were washed three times with PBS alone, and then 100 μL/well of HRP/anti-M13 monoclonal conjugate (GE Healthcare) diluted 1:2500 in PBS containing BSA [2% (w/v)] was added to each well followed by incubation at room temperature for 1 h with agitation (∼100 rpm). The wells were washed three times with PBS−Tween-20 and three times with PBS alone, and then SureBlue Reserve TMB Microwell Peroxidase Substrate from KPL (Gaithersburg, MD) was added to each well (100 μL/well). The reaction was stopped by adding 1 N HCl (100 μL/well) after 2−7 min (i.e., when there was a clear difference between the observed color in the sample and control wells). Absorbance at 450 nm was measured with a microplate reader (SpectraMax 190) from Molecular Devices (Sunnyvale, CA). Clones were identified as potential antigen binders if the absorbance was at least ≥0.2 and at least 2-fold higher than the background. The DNA sequence for each of the strong antigen binders was determined using a BigDye Terminator v3.1 Cycle Sequencing kit from Applied Biosystems (Foster City, CA) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's recommended procedures. The sequencing primers were designed to anneal to the vector sequences flanking the scFv gene. The sequences of the primers were GAAATACCTATTGCCTACGG and GAACCAGAGCCACCACCCTAC.

Expression and Purification of scFv. The scFv gene for the strongest binder was subcloned into the bacterial expression vector pET30a from Novagen (Madison, WI) at the *Nde*I and *Xho*I restriction enzyme sites so that the scFv gene product would be expressed with a polyhistidine tag at the C terminus. Competent Rosetta (DE3) pLysS *E.coli* cells (Novagen) were transformed with the scFv-pET30a construct and used for bacterial overexpression for subsequent scFv purification. Five milliliters of Luria−Bertani (LB) medium containing 50 μg/mL kanamycin and 30 μg/mL chloramphenicol (LB-KC) was inoculated with a single colony of the transformed *E. coli*. The culture was incubated overnight at 37 °C with agitation at 250 rpm, and 1 mL of the overnight culture was used to inoculate fresh 250 mL aliquots of LB-KC. The culture was incubated at 37 °C with agitation at 250 rpm until $OD_{600nm} = 0.4-0.6$ and scFv expression was induced with IPTG (1 mM final concentration). The induced bacteria were incubated for 3 h at 37 °C with agitation at 250 rpm and then centrifuged (10000*g*, 10 min, 4 °C). The bacterial pellet was resuspended in (10 mL/250 mL of culture) buffer 1 (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, pH 7.0). The bacteria were sonicated on ice and centrifuged (10000*g*, 10 min, 4 °C), and the supernatant was removed. Due to the expression of the scFv in the insoluble protein fraction, the pellet was resuspended in (10 mL/250 mL of culture) buffer 2 (100 mM $NaH_2PO_4 \cdot H_2O$, 10 mM Tris, 8 M urea, 5 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). The sample was stirred with a magnetic stir bar on a magnetic stir plate at room temperature for 1 h and then centrifuged (10000*g*, 10 min, 4 °C). The supernatant was added to (12 mL/250 mL culture) Ni-NTA agarose (Qiagen), which had been equilibrated with buffer 2, and the solution was incubated overnight at room temperature. After collection of the flowthrough, the Ni-NTA agarose was washed first with 100 mL of wash buffer 1 (100 mM $NaH_2PO_4 \cdot H_2O$, 10 mM Tris, 8 M urea, 20 mM imidazole, pH 8.0) and then with wash buffer 2 (100 mM $NaH_2PO_4 \cdot H_2O$, 10 mM Tris, 8 M urea, 200 mM imidazole, pH 8.0). Approximately 100 mL of wash buffer 2 eluant containing scFv was collected and then analyzed by gel electrophoresis in a 13% SDS-PAGE gel stained with Coomassie Brilliant Blue R250 from Sigma-Aldrich (Oakville, Canada). To refold the denatured purified scFv, the collected wash buffer 2 eluant was dialyzed sequentially against three buffers essentially as described in ref *20*. The first dialysis was performed overnight against 5 mM reduced glutathione and 0.5 mM oxidized glutathione. The second dialysis was performed for 66 h against 50 mM Tris, pH 7.5, and 0.4 M L-arginine followed by dialysis for 48 h against 0.05× PBS, pH 7.5. All dialysis steps were performed at 4 °C using 6 L of dialysis buffer. The soluble scFv was lyophilized to concentrate and then resuspended in sterilized water (1:20 volume).

Biotinylation of scFv. The protocol used for biotinylation of purified scFv was modified from the method described in ref *24* and was

performed using biotinamidohexanoic acid-3-sulfo-*N*-hydroxysuccinimide ester (long-arm biotin; Sigma-Aldrich). Briefly, 1 mg of scFv (in PBS) was incubated with 20 μL of 3 μg/μL biotin (in PBS) for 1 h at room temperature with periodic mixing. Ten microliters of 100 mg/mL glycine, which had been prepared in water, was added, and the solution was incubated for 10 min at room temperature to stop the biotinylation reaction. The scFv solution was dialyzed against PBS at pH 7 and 4 °C to remove free biotin.

Determination of Specificity of scFv. *Alternaria brassicae* (Berk.) Sacc. and *Leptosphaeria maculans* (Desmaz.) Ces. and De Not. were grown on solid V8 juice media [20% (v/v) V8 juice, 5% (w/v) rose bengal, 0.3% (w/v) $CaCO_3$, 2% (w/v) agar], and *Botrytis cinerea* (Pers.: Fr.) and *S. sclerotiorum* were grown on potato dextrose agar (PDA; Difco) plates. Each of the fungi was cultured on the respective agar plates from pure fungal stocks that were stored in liquid nitrogen as glycerol stocks. Fungal growth and preparation were performed aseptically to avoid contamination. *A. brassicae* was grown in the dark for approximately 2 weeks, whereas *L. maculans* was grown under light from cool white fluorescent tubes (12 h photoperiod) for approximately 2 weeks. *B. cinerea* and *S. sclerotiorum* were grown under continuous light from cool white fluorescent tubes for 5−7 days. All fungi were grown at room temperature. The fungal mycelia collected from the plates were ground in separate mortars containing PBS for a final concentration of 0.5 mg/mL. Eight-well Flat Bottom ImmunoModule wells were coated with PBS (control) or a fungal mycelia/PBS solution (100 μL/well) and incubated overnight at 4 °C. The wells were washed three times with PBS, blocked with 3% (w/v) BSA in PBS (200 μL/well), and incubated for 2 h at room temperature with agitation at ∼100 rpm. The wells were washed three times with PBS, and biotinylated scFv (1 ng/μL) in 3% (w/v) BSA/PBS was added to each well (100 μL/well). Following incubation for 1 h at room temperature with agitation at ∼100 rpm, the wells were washed five times with PBS containing 0.05% (v/v) Tween-20 and six times with PBS. ImmunoPure Streptavidin−HRP from Pierce (Rockford, IL) was diluted in BSA [final concentration 0.1 μg/mL in 3% (w/v) BSA/PBS], added to each well (100 μL/well), and incubated for 1 h at room temperature with agitation at ∼100 rpm. The wells were washed with PBS−Tween and PBS as before, SureBlue Reserve TMB Microwell Peroxidase Substrate was added (100 μL/well), and the colorimetric reaction was stopped with 1 N HCl (100 μL/well) after 2−7 min (i.e., when there was a clear difference between the observed color in the sample and control wells). The absorbance at 450 nm was measured with a microplate reader (SpectraMax 190). The experiment was performed twice, and each time ELISA measurements were done in triplicate. ELISA results were analyzed using the GLM procedure for ANOVA followed by Duncan's multiple-range test ($p < 0.05$) with SAS software version 9.1 (SAS Institute Inc., Cary, NC).

Histological Examination of Sclerotinia−scFv Interaction. Warm (45 °C) 1% (w/v) agar was poured onto *S. sclerotiorum* mycelia grown on PDA plates, which were then cut into small (10 × 5 × 2 mm) blocks and fixed at 4 °C overnight in 50 mM phosphate buffer containing 4% (v/v) paraformaldehyde. The tissue blocks were dehydrated in a graded ethanol series, changed to toluene, and infiltrated with Paraplast employing Fisher Histomatic Tissue Processor (model 166) from Fisher Scientific (Pittsburgh, PA). Sections were cut (6 μm thickness) with an AO Rotary microtome (Spencer 820), affixed to slides, deparaffinated with toluene, and rehydrated to water with a graded ethanol series. Endogenous peroxidase activity was blocked by incubating slides overnight with 40% (v/v) methanol in PBS containing 0.3% (v/v) hydrogen peroxide at 4 °C (*25*). The sections were rinsed with PBS (2 × 5 min each) and blocked with 3% (w/v) BSA in PBS for 2 h, after which the sections were rinsed once with PBS containing 0.05% (v/v) Tween-20 for 5 min and incubated with biotinylated scFv (60 μg in 600 μL of PBS−Tween-20) for 90 min. The sections were rinsed three times (10 min each) with PBS−Tween-20 and incubated with ImmunoPure Streptavidin−HRP for 90 min. Control sections were untreated (i.e., no scFv or streptavidin−HRP) or incubated with scFv but no streptavidin−HRP or incubated with streptavidin−HRP but no scFv. The sections were rinsed with PBS−Tween-20 (three times, 10 min each), and peroxidase activity was detected with the TMB peroxidase substrate from Vector Laboratories (Burlingame, CA) according to the manufacturer's instructions. After approximately 10 min, sections were dehydrated with a graded ethanol series, followed by toluene and mounted with Parmount, and a microscopic cover glass was placed over them. The sections were viewed with a Leica DM *RXA* microscope from Leica Microsystems (Wetzlar, Germany) for the presence or absence of stain and photographed with an Optronics digital camera employing Macrofire software from Optronics (Goleta, CA).

Dimerization of scFv. The scFv gene was subcloned into the scFv-pET30a bacterial expression construct described earlier at the *Xba*I and *Nde*I restriction enzyme sites. A DNA segment encoding a short amino acid sequence $(G_4S)_2$ separated the two scFv genes. The scFv dimer gene was subcloned into the bacterial expression vector pET28a at the *Nco*I and *Xho*I restriction enzyme sites so that the scFv dimer gene product would be expressed with a polyhistidine tag at the C terminus. Expression, purification, and biotinylation of the scFv-dimer were performed as previously described.

ELISA-Based Comparison of scFv and scFv Dimer. Eight-well Flat Bottom ImmunoModule wells were coated with PBS (control) or different concentrations of *S. sclerotiorum* mycelia ground in PBS (100 μL/well; 0.5, 0.05, and 0.005 mg/mL). The ELISA was performed as described earlier for the determination of specificity of scFv. The experiment was performed twice, and each time ELISA measurements were done in triplicate. ELISA results were analyzed using the GLM procedure for ANOVA followed by Duncan's multiple-range test ($p < 0.05$) with SAS software version 9.1 (SAS Institute Inc.).

Detection of Mycelia in Inoculated Canola Petals. Canola petals collected from *Brassica napus* (Westar) were surface sterilized in 70% (v/v) ethanol for 1 min followed by 2.6% (v/v) sodium hypochlorite containing 0.05% Tween-20 for 5 min. The petals were rinsed in sterile water and aseptically placed along the margin of actively growing *S. sclerotiorum* mycelia on PDA plates, which is a protocol modified from ref *15*. As a control, surface-sterilized petals were placed on PDA plates containing no mycelia. The plates were incubated at room temperature for 3 days, and then the petals were collected using sterile forceps and divided into 6 groups of 20 with differing percentages of petals inoculated with mycelia (0, 5, 25, 50, 75, and 100%). Each group of petals was ground in PBS (1 mL) using separate mortars and pestles, and the resulting slurries were centrifuged (21000*g*, 10 min, 4 °C) to remove plant tissue. Eight-well Flat Bottom ImmunoModule wells were coated with PBS (control) or petal supernatant (100 μL/well) and incubated overnight at 4 °C. The wells were washed three times with PBS, blocked with 3% (w/v) BSA in PBS (200 μL/well), and incubated for 2 h at room temperature with agitation (∼100 rpm). The wells were washed three times with PBS, and biotinylated scFv dimer (5 ng/μL) in 3% (w/v) BSA−PBS was added to each well (50 μL/well). Following incubation for 1 h at room temperature with agitation (∼100 rpm), the wells were washed five times with PBS containing 0.05% (v/v) Tween-20 and six times with PBS. ImmunoPure Streptavidin−HRP was diluted in BSA [final concentration = 0.1 μg/mL in 3% (w/v) BSA/PBS], added to each well (50 μL/well), and incubated for 1 h at room temperature with agitation at ∼100 rpm. The wells were washed with PBS−Tween and PBS as before, SureBlue Reserve TMB Microwell Peroxidase Substrate was added (50 μL/well), and the colorimetric reaction was stopped with 1 N HCl (50 μL/well) after 2−7 min (i.e., when there was a clear difference between the observed color in the sample and control wells). Absorbance at 450 nm was measured with a microplate reader (SpectraMax 190). The experiment was performed twice, and each time ELISA measurements were done in triplicate. ELISA results were analyzed using the GLM procedure for ANOVA followed by Duncan's multiple-range test ($p < 0.05$) with SAS software version 9.1 (SAS Institute Inc.).

RESULTS AND DISCUSSION

Isolation of scFv Strong Binders. The immunization of mice with mycelial antigens resulted in polyclonal antibodies as detected in ELISA. The spleen from the best responder was used to extract total RNA for developing a phage display immune library for selection of potential scFv binders. The initial phage library contained approximately 10^{11} phage particles. Following two to four rounds of panning, phage ELISAs were

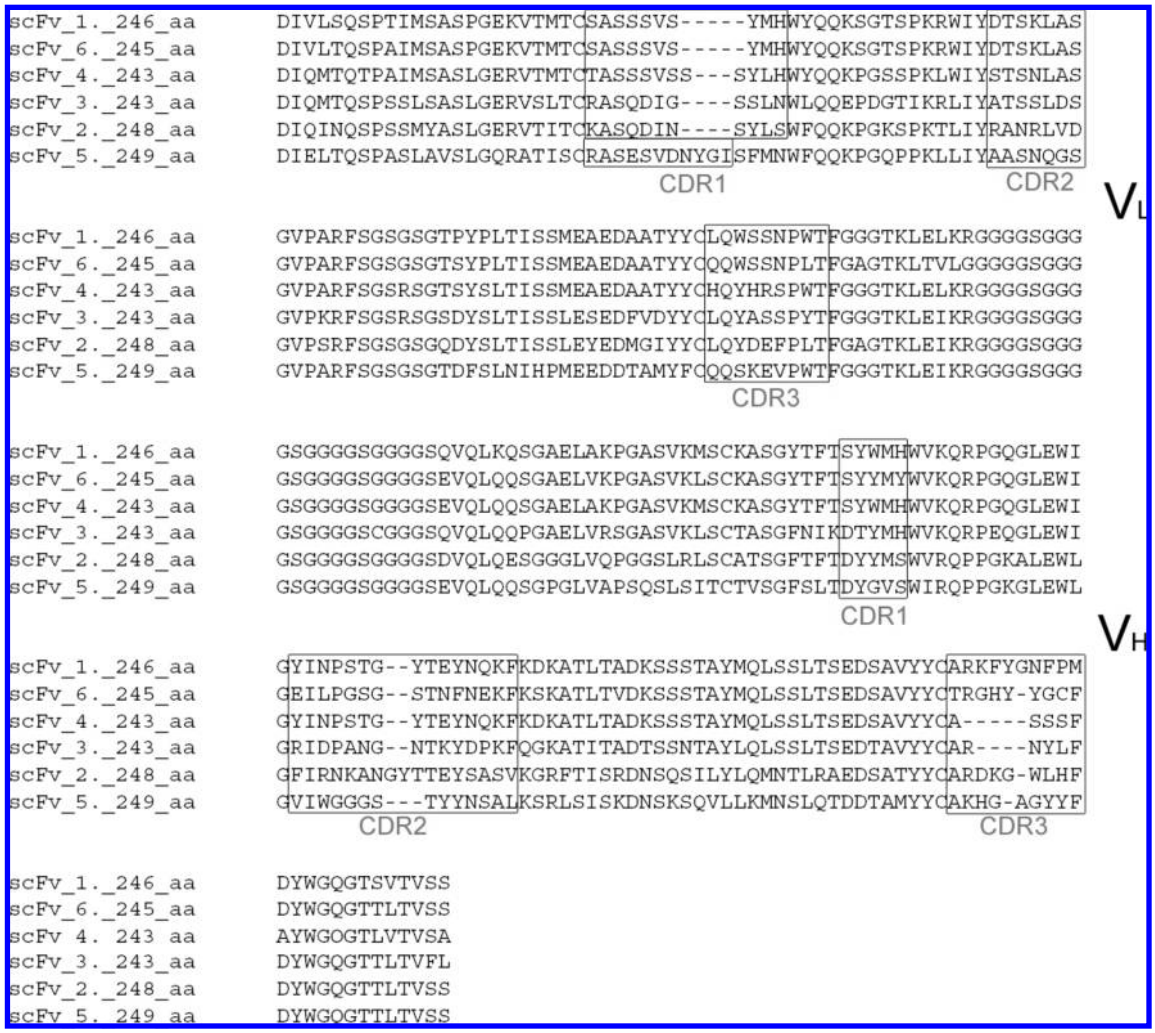

Figure 1. Multiple amino acid sequence alignments of the six isolated scFv strong binders to *S. sclerotiorum* mycelia. The scFv antibody was constructed such that the final orientation was $V_L-(G_4S)_4-V_H$. The CDRs of the variable domains are indicated. The number of amino acids in each of the isolated scFv antibodies is also provided.

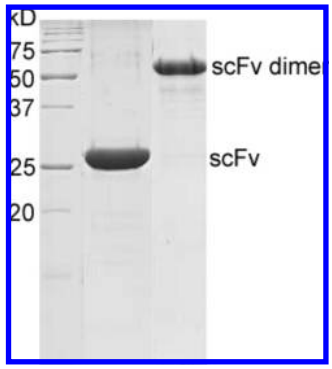

Figure 2. Image of a Coomassie Blue R250-stained 13% SDS-PAGE gel showing purity of scFv and scFv dimer following immobilized metal affinity chromatography. The expected sizes of the scFv and scFv dimer were approximately 27 and 55 kDa, respectively.

performed, resulting in the identification of six potential scFv strong binders. The clone for the strongest binder (scFv_1) produced an absorbance (450 nm) of 0.360 in the phage ELISA. The DNA sequences of the isolated clones were translated, and their amino acid sequence alignments are shown in **Figure 1**. Two of the clones encoded scFv antibodies that consisted of 243 amino acids, whereas the remaining scFv antibodies consisted of 245, 246, 248, and 249 amino acids. Although there are a number of differences in the amino acid sequences within the complementarity-determining regions (CDR) in both the V_L and V_H antibody domains among the various clones, there are also a number of amino acid sequence differences within the flanking regions. The decision to construct the scFv in the orientation $V_L-(G_4S)_4-V_H$ rather than $V_H-(G_4S)_4-V_L$ was based on the fact that several scFv antibodies in the $V_L-(G_4S)_4-V_H$ configuration have been generated by others and showed high affinity for their target antigens (*20, 22, 23*). The scFv gene for the strongest binder was selected for subcloning into a bacterial expression vector for subsequent experiments including the generation of a dimeric molecule.

Expression and Purification of scFv. The cDNAs encoding scFv and scFv dimer were subcloned into bacterial expression vectors, which were transformed into competent *E. coli* expression cells. After 3 h of induction, the bacterial cells were harvested and lysed, and the soluble and insoluble protein fractions were collected. Because the expression of scFv and scFv dimer was almost exclusively within the insoluble protein fraction, the purification necessitated the use of denaturing buffers. After elution of the scFv and scFv dimer from the Ni-NTA resin, the protein was refolded using stepwise dialysis. Changes to the induction conditions were attempted to increase soluble scFv and scFv dimer expression; however, the use of different concentrations of IPTG, induction at different tem-

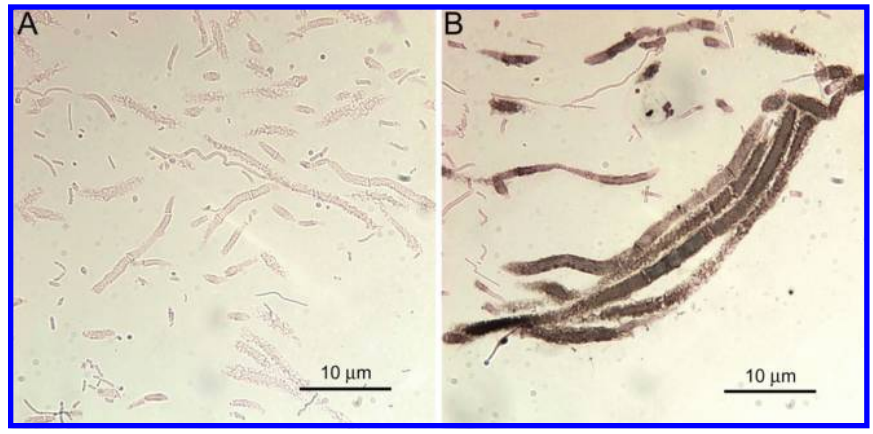

Figure 3. Light microscopy results demonstrating the ability of the scFv to bind *S. sclerotiorum* (**B**). The biotinylated scFv was detected using HRP-conjugated streptavidin. The appearance of the mycelia without the addition of scFv or HRP-conjugated streptavidin is shown (**A**) and served as a control.

peratures, and use of commercially available kits for periplasmic localization did not affect the solubility of the expressed scFv or scFv dimer (data not shown). **Figure 2** shows a Coomassie Blue R250-stained 13% SDS-PAGE gel indicating the level of purity achieved during the purification of scFv and scFv dimer.

During the refolding dialysis, a considerable amount of precipitation was observed in the dialysis tubing, particularly during the second dialysis step in 50 mM Tris, pH 7.5, and 0.4 M L-arginine buffer. Higher concentrations of scFv or scFv dimer increased precipitation, which could not be reduced even at scFv concentrations as low as 30 μg/mL. Despite the loss of protein due to precipitation, protein concentration following lyophilization indicated that the final yield of refolded, soluble antibody was approximately 10−12 mg/L of bacterial culture for both scFv and scFv dimer. The fact that the yield was approximately 2−3-fold less than what has been reported by others was likely due to the observed precipitation (*26, 27*).

Microscopy and Specificity of scFv. In lieu of the identification of the antigen recognized by the scFv, light microscopy experiments were performed to verify that the isolated scFv strong binder does, in fact, recognize and bind *S. sclerotiorum* mycelia, and the resulting images are shown in **Figure 3**. The appearance of the fungal mycelia without the addition of the biotinylated scFv or the HRP-conjugated streptavidin is shown in **Figure 3A**, whereas the appearance of the mycelia upon addition of the scFv and streptavidin−HRP is shown in **Figure 3B**. There was no staining observed in either of the two other controls, (1) scFv but no streptavidin−HRP or (2) streptavidin−HRP but no scFv (data not shown). The results from the controls along with the fact that endogenous peroxidase was blocked prior to the addition of scFv led to the conclusion that the observed staining in **Figure 3B** was the result of scFv binding to the *S. sclerotiorum* mycelia. Although the identification of the precise antigen recognized by the scFv is unknown, the microscopy result clearly shows that the scFv does have affinity for some component of the fungal mycelia.

Although the microscopy result confirmed that the isolated scFv is capable of binding to *S. sclerotiorum* mycelia, it did not provide any information regarding the specificity of the scFv. A conclusion could not be made in terms of whether or not the scFv could bind to fungi other than *S. sclerotiorum*, so an ELISA was performed to determine if the scFv could recognize and bind other phytopathogenic fungi that may be present in canola fields. The comparison of the absorbance values generated for *A. brassicae*, *L. maculans*, *B. cinerea*, and *S. sclerotiorum* shown in **Figure 4** indicates that the isolated scFv differed in its affinity

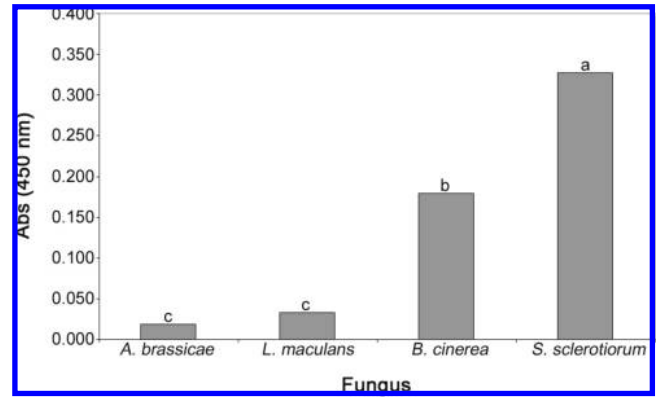

Figure 4. ELISA-based comparison of scFv binding to different phytopathogenic fungi showing that the antibody binds to *S. sclerotiorum* with some cross-reactivity to the genetically similar fungus *B. cinerea*. There does not appear to be any appreciable affinity for *A. brassicae* or *L. maculans*. Data points with different letters are significantly different according to ANOVA analysis by SAS ($p < 0.05$).

for the fungi tested. Controls were performed to verify that the observed color reaction was the result of biotinylated scFv binding to fungal mycelia and not the result of endogenous fungal peroxidases reacting with the SureBlue Reserve TMB Microwell Peroxidase Substrate or the result of streptavidin−HRP binding to the fungal mycelia (data not shown). There does not appear to be any substantial affinity for either *A. brassicae*, which is the causal agent of blackspot of canola (*28*), or *L. maculans*, which causes blackleg disease of canola (*29*). Although the highest absorbance values were observed for *S. sclerotiorum*, there does appear to be some cross-reactivity with *B. cinerea*. This result is perhaps not unexpected and may be explained by the fact that *Botrytis* is genetically similar to *Sclerotinia*, and therefore the antigen that is being recognized may be common to the two phytopathogenic fungi (*30*). A similar cross-reactivity between the two taxonomically related fungi was previously observed for polyclonal antibodies that were used in ELISA-based experiments (*16*). *B. cinerea* has been observed to infect flower petals as part of its disease cycle on other plants (*31*); however, gray mold caused by *B. cinerea* is rarely a problem in canola production in western Canada (*5*), so it is typically not observed on petals or on other plant tissues of canola. This does not preclude the possibility that *B. cinerea* may be present on canola petals in the field, and this possibility must be addressed before a viable diagnostic test can be developed.

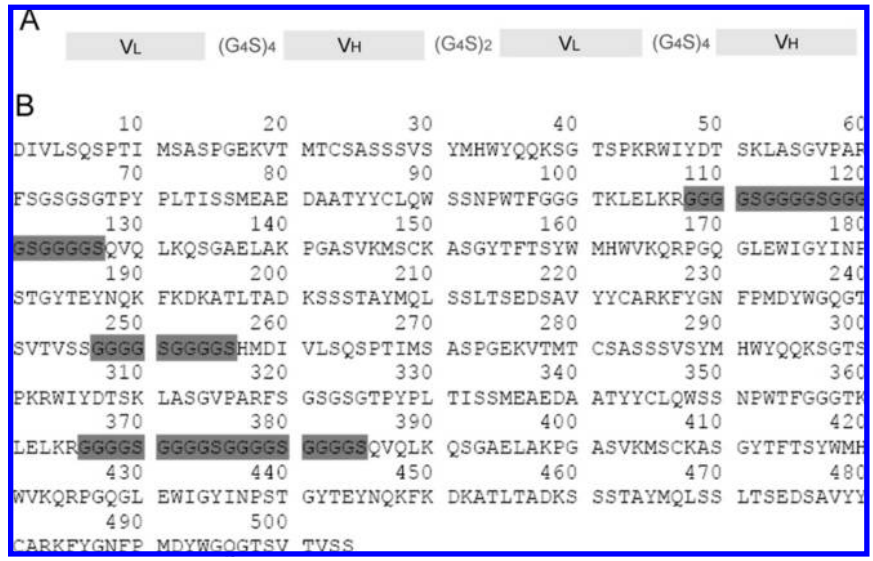

Figure 5. Schematic representation (**A**) and amino acid sequence (**B**) of the scFv dimer. The highlighted amino acids represent the peptide linker separating the monomeric scFv antibodies $(G_4S)_2$ or the peptide linker separating the V_L and V_H antibody domains $(G_4S)_4$.

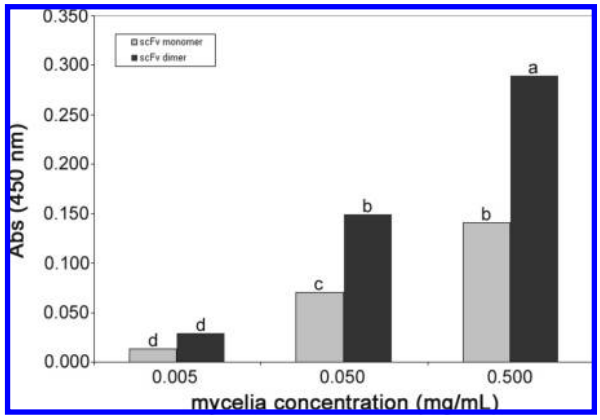

Figure 6. ELISA-based comparison of scFv monomer and scFv dimer binding to *S. sclerotiorum* mycelia. At each of the mycelia concentrations tested, the absorbance value for scFv dimer was approximately 2-fold higher than that for scFv monomer. Data points with different letters are significantly different according to ANOVA analysis by SAS ($p < 0.05$).

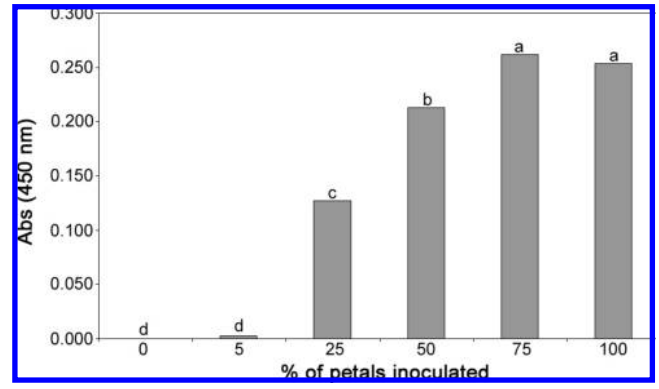

Figure 7. Detection of canola petals inoculated with *S. sclerotiorum* mycelia using scFv dimer. As the percentage of petals inoculated with mycelia increases, the absorbance value obtained from an ELISA-based assay also increases. Data points with different letters are significantly different according to ANOVA analysis by SAS ($p < 0.05$).

Comparison of scFv and scFv Dimer. To improve the binding of the scFv antibody to *S. sclerotiorum* mycelia, the antibody fragment was dimerized to create a molecule consisting of two scFv fragments linked by a short polypeptide linker (**Figure 5**). The results of the ELISA-based comparison of the scFv and the scFv dimer are shown in **Figure 6**, which reveals that at each of the mycelia concentrations tested, the absorbance value for the scFv dimer is almost exactly 2-fold higher than for the scFv. This result can be explained by the fact that the dimer contains two antigen-binding sites as compared to the single binding site within the scFv, resulting in higher absorbance values.

Detection of Mycelia in Inoculated Canola Petals. The ability of our scFv dimer to detect mycelia in plant tissue was evaluated using ELISA. **Figure 7** illustrates that as the percentage of canola petals inoculated with *S. sclerotiorum* mycelia increases, there is an increase in the absorbance value. This result demonstrates the potential for the scFv dimer to be used in a diagnostic test such as an antibody-based "point of care" (POC) test that can be utilized by canola growers to determine the likelihood of a *S. sclerotiorum* infestation. The immunoswab detection test described in ref *32* is an example of the type of POC diagnostic test that might be developed using the scFv dimer. A potential advantage of a simple and sensitive POC test to detect the presence of *S. sclerotiorum* mycelia over currently utilized petal test kits is the speed at which the results might be generated. Petal test kits require that the canola petals collected from random locations within a field be placed on appropriate growth media followed by an observation of the resulting fungal growth to determine if *S. sclerotiorum* was present on the canola petals (*33*). Sufficient growth of the various fungi present on the petals would likely require a few days before a final analysis of the growth media can be made; however, a POC test could provide results more quickly.

Although the ELISA results generated in this study are promising for the development of a diagnostic test, it is acknowledged that more work is required to fully validate the utility of the scFv dimer in a POC test. We are not suggesting that currently utilized effective tools for risk assessment, such as the petal test kit, and disease management will become obsolete with the introduction of a POC-type detection system. Instead, when developed, the POC test would provide an additional tool to aid in decision-making regarding fungicide application. Future experiments that are required for the development of a viable POC diagnostic assay will involve the use of field-collected canola petals and a quantitative test that correlates with the level of *S. sclerotiorum* infestation in canola

fields and disease incidence. Merely determining the presence or absence of the fungus in a petal sample would likely not provide enough information to enable canola growers to make an educated decision on whether an application of fungicide is necessary to effectively control *S. sclerotiorum* infection. Furthermore, the affinity of the scFv dimer for *S. sclerotiorum* may need to be increased for any future diagnostic assay to improve the sensitivity of the test. Increasing the affinity of the scFv dimer may be accomplished using site-directed mutagenesis or random mutagenesis of the scFv gene.

To our knowledge, this is the first report of a scFv dimer with affinity for *S. sclerotiorum* that has the potential for use in a diagnostic assay. The development of a POC diagnostic test for this ubiquitous phytopathogenic fungus could provide farmers with a novel tool to be utilized as part of an effective and viable disease management strategy. The potential agricultural and economic benefits resulting from the production of a new diagnostic test could be significant and warrant continued efforts in the development of such a test.

ABBREVIATIONS USED

BSA, bovine serum albumin; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria−Bertani; LB-KC, Luria−Bertani−kanamycin and chloramphenicol; Ni-NTA, nickel nitrilotriacetate; PBS, phosphate-buffered saline; PCR, Polymerase Chain Reaction; PDA, potato dextrose agar; POC, point of care; scFv, single-chain variable fragment; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; V_L, variable light; V_H, variable heavy; 2× YT-GC, 2× YT−glucose and chloramphenicol.

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