Characterization of the *VisdhC* and *VisdhD* Genes in *Venturia inaequalis*, and Sensitivity to Fluxapyroxad, Pydiflumetofen, Inpyrfluxam, and Benzovindiflupyr

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Abstract

Succinate dehydrogenase inhibitors (SDHI) are an important class of fungicides for management of apple scab, especially as resistance to other classes of fungicides has become prevalent in the northeastern United States. Considering their single-site mode of action, there is high risk of resistance development to SDHI fungicides. Such risk mandates the need for proper monitoring of shifts in population sensitivity. This study aims to provide a means for phenotypic and genotypic characterization of SDHI fungicide resistance for *Venturia inaequalis*, the causal agent of apple scab. To complement the published sequence of *VisdhB*, target genes *VisdhC* and *VisdhD* were identified using sequences of homologous genes in other fungal organisms and a draft genome of *V. inaequalis*. Using mycelial growth and conidial germination assays, baseline sensitivities and cross sensitivities of *V. inaequalis* were determined for several SDHI fungicides. Mean baseline EC₅₀ values for conidial germination of benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam were found to be 0.0021, 0.0284, 0.014, and 0.0137 $\mu\text{g ml}^{-1}$, respectively. Mean baseline EC₅₀ values for mycelial growth of

benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam were found to be 0.0575, 0.228, 0.062, and 0.0291 $\mu\text{g ml}^{-1}$, respectively. A significant and positive correlation in sensitivity was found between benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam as well as penthiopyrad and fluopyram, with the highest correlation between benzovindiflupyr and penthiopyrad for mycelial inhibition of *V. inaequalis* ($r = 0.950$, $P < 0.001$). For inhibition of conidial germination, the highest correlation was observed between penthiopyrad and fluopyram ($r = 0.775$, $P < 0.001$). Furthermore, the sequences of the *VisdhC* and *VisdhD* genes were identified and characterized for baseline isolates of *V. inaequalis*. Residues of similar position to mutations found in other systems that confer resistance to SDHI fungicides were identified in baseline isolates, but no mutations were identified in baseline isolates or those previously exposed to SDHI fungicides. This study will serve as a reference for future monitoring of resistance to SDHI fungicides in *V. inaequalis* at both a phenotypic and genotypic level.

Apple scab, caused by the ascomycete fungus *Venturia inaequalis* (Cooke) G. Winter, is one of the most economically devastating diseases of apple (MacHardy 1996). Conventional management relies on multiple fungicide applications throughout the growing season due to the lack of commercially popular cultivars with host resistance (MacHardy 1996; Merwin et al. 1994). Increased use of single-site fungicides has resulted in subsequent selection for resistant isolates and a consequential loss of fungicide efficacy, as seen with widespread resistance to quinone outside inhibitors (QoI) (Frederick et al. 2014; Lesniak et al. 2011; Villani and Cox 2014). Recently, new chemistries in the class of succinate dehydrogenase inhibitors (SDHIs) have been registered for disease control in apples, and with these new products come new opportunities for effective control of apple scab. While SDHI fungicides were first commercialized in the 1960s, these original chemistries were not widely used in apple due to the narrow range of activity restricted to basidiomycetes (Sierotzki and Scalliet 2013; Xiong et al. 2015). Next-generation SDHI fungicides have high efficacy on a broader spectrum extended to ascomycete fungi, including the apple scab pathogen, by targeting complex II of the mitochondria and interfering with cellular

respiration (Hagerhall 1997; Sierotzki and Scalliet 2013; Villani et al. 2016).

The high intrinsic activity and specificity for their target presents a high risk of resistance development to SDHI fungicides (Avenot and Michailides 2010; Hu et al. 2016). Additionally, as other methods of chemical control become obsolete due to resistance development, there will be a heavier reliance on the use of the newer SDHI fungicides, limiting options for chemical rotation (Chapman et al. 2011; Cox 2015). This highlights the necessity of sustainable use of SDHI fungicides with emphasis on resistance monitoring to protect the durability of this class of fungicides. While there are currently no known populations of *Venturia inaequalis* with resistance to SDHI fungicides, resistance to SDHIs has been noted in other fungal species, including *Botrytis cinerea* (Fernández-Ortuño et al. 2017), *Stagonosporopsis citrulli* (syn. *Didymella bryonia*) (Thomas et al. 2012), *Alternaria solani* (Mallik et al. 2014), *Sclerotinia sclerotiorum* (Wang et al. 2015), and *Alternaria alternata* (Avenot et al. 2009). These observations of resistance necessitate the availability of careful monitoring protocols for *V. inaequalis* to best understand resistance development and ensure permanency of SDHI fungicides in this system.

The *sdh* target gene is composed of four subunits; a flavoprotein (SDHA), an iron-sulfur unit (SDHB), and two membrane-bound units (SDHC and SDHD), but SDHI fungicides only come in direct binding contact with the SDHB, SDHC, and SDHD subunits, otherwise known as the ubiquinone binding pocket (Sierotzki and Scalliet 2013). Mutations conferring resistance can therefore occur in any of the three subunits where SDHs directly bind. The most common of these mutations is typically found in the SDHB subunit, where a critical histidine residue is replaced by arginine, leucine, or tyrosine to confer resistance (Sierotzki and Scalliet 2013). At this time, only the *VisdhB* gene has been characterized for *V. inaequalis* (Villani et al. 2016). While several mutations associated with SDHI resistance have been identified in the SDHB subunit in other phytopathogenic

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fungi, this does not explain all incidences of resistance. The H134R mutation and the D123E mutation have been well documented for *Alternaria alternata* in the SDHC and SDHD subunit, respectively (Avenot et al. 2009). This illustrates the importance of elucidating the sequence of the *VisdhC* and *VisdhD* genes for resistance monitoring.

Following extended exposure to SDHI fungicides, shifts in sensitivity have been observed in other fungal pathogens, including *B. cinerea* (Hu et al. 2016) and *Monilinia fructicola* (Amiri et al. 2010), illustrating the importance of monitoring phenotypic changes in sensitivity. To most accurately assess changes in sensitivity, it is first necessary to determine effective concentrations of SDHI fungicides at which 50% of growth is inhibited (EC_{50} values) for baseline isolates. Baseline sensitivities have previously been determined for the registered SDHI fungicides penthiopyrad, fluopyram, and benzovindiflupyr (Villani et al. 2016), leaving the baseline sensitivity of recently registered fluxapyroxad and the forthcoming inpyrfluxam and pydiflumetofen. Furthermore, due to the similarity in chemistries and modes of action, it is appropriate to address concerns of cross-sensitivity between all the SDHI fungicides.

The objectives of this study were to (i) clone the remaining target genes of SDHI fungicides: *VisdhC* and *VisdhD*, (ii) determine the baseline sensitivity to four SDHI active ingredients: fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr, and (iii) determine cross-sensitivity among all six SDHI fungicides that are currently registered or in development for use on apple.

Materials and Methods

Isolate collection. Thirty-five baseline isolates with no previous exposure to modern fungicides were collected from apple trees in Romulus, Geneva, and Waterloo, NY, from 2013 to 2017 to use for characterization of baseline sensitivity (Villani et al. 2015). To compare the baseline with a population exposed to SDHI fungicides, >30 additional isolates were collected from a 20-year-old research orchard in Geneva, NY, consisting of Empire and Cortland apples on M.9/M.111 interstem rootstocks. During the growing season prior to collection, trees were subjected to four applications of Sercadis (BASF, Research Triangle Park, NC), a formulated product containing fluxapyroxad. Sercadis was applied at rate of 256.2 ml/ha dilute to run-off with a AA2 GunJet handgun (TeeJet Technologies, Glendale Heights, IL) at 551 kPa. Fungicides were applied in coordination with infection periods at pink, bloom, first cover (10 days after petal fall), and second cover (approximately 24 days after petal fall) with multisite protectants applied at green tip and petal fall. During June 2016 and 2017, a minimum of 15 leaves with apple scab lesions were randomly collected from each of the treatment replications. In addition, symptomatic leaves were collected from the baseline locations sampled previously (Villani et al. 2015). From these leaves, isolated individual lesions arising from a single ascospore infection event (Köller et al. 2004; MacHardy and Gadoury 1989) were selected for monospore isolation. Conidia of *V. inaequalis* were obtained as previously described (Frederick et al. 2014; Villani et al. 2015), by first excising individual lesions with a 5-mm-diameter cork borer. Lesions were then placed in 1.2 ml deionized water and shaken for 60 s to dislodge and suspend conidia in water at an approximate concentration ranging from 10^2 to 10^3 conidia ml^{-1} (Villani et al. 2016). The leaf discs were removed and discarded from the tubes with a sterile toothpick. Conidial suspensions were stored at $-20^{\circ}C$ for up to 5 years prior to fungicide evaluations.

Identification of *VisdhC* and *VisdhD* genes. The draft genome sequence of *V. inaequalis* for isolate 3a-27-17, as described by Villani et al. (2016), was used to identify areas of high homology with closely related fungal organisms. Accessions from NCBI GenBank for the *sdhC* and *sdhD* genes from *Alternaria alternata* (accession numbers KJ426267 and KJ426275, respectively) and *Botryotinia fuckeliana* (accession numbers GQ253443 and GQ253441, respectively) were used to locate putative genes in *V. inaequalis*. A genome search in the assembly of isolate 3a-27-10 for homologous contiguous areas was completed with the local CLC Main Workbench 7 (version 7.8.1, Qiagen Bioinformatics, Redwood City, CA) BLAST

function. Primers were then designed to target putative upstream and downstream sequences of *VisdhC* and *VisdhD* genes (Table 1).

To amplify putative *VisdhC* and *VisdhD* genes, DNA was extracted from baseline isolate 10-3-14, which had been cultured on potato dextrose agar (PDA) (Difco Laboratories Inc., Detroit, MI) and incubated at room temperature (20 to $23^{\circ}C$) for 4 weeks. To extract genomic DNA (gDNA), approximately 50 mg of mycelium was excised from the agar and ground in liquid nitrogen using a mortar and pestle. An Omega BioTek E.Z.N.A Plant DNA Kit (Omega Bio-Tek, Norcross, GA) was used to isolate total gDNA in accordance with the manufacturer's instructions. To locate introns and coding regions, an RNA extraction was also completed for the same isolate. Briefly, isolate 10-3-14 was grown and tissue was lysed as described above. RNA was extracted using an Omega BioTek E.Z.N.A Plant RNA Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. DNA contamination was removed using TURBO DNA-free Kit (Ambion by Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Complementary DNA (cDNA) was generated from RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA) following the manufacturer's instructions.

Both extracted cDNA and gDNA were then amplified through polymerase chain reaction (PCR). Primer pairs *VisdhC*-106F/*VisdhC*-200R and *VisdhC*1F/*VisdhC*654R were used to amplify gDNA of the *VisdhC* gene, while primer pairs *VisdhD*-139F/*VisdhD*713R and *VisdhD*-139F/*VisdhD*695R were used to amplify the *VisdhD* gene. cDNA of *VisdhC* and *VisdhD* was amplified using primer pairs *VisdhC*1F/*VisdhC*654R and *VisdhD*1f/*VisdhD*713R, respectively. A PCR was completed at the following cycles using a T100 Thermal Cycler (Bio-Rad Laboratories Inc.): 2 min at $94^{\circ}C$, 30 cycles of 30 s at $94^{\circ}C$, 30 s at specified annealing temperature (Table 1), and 45 s at $72^{\circ}C$, followed by a final extension at $72^{\circ}C$ for 7 min. For each primer pair, 25- μ l reactions were prepared composed of the following: 12.5 μ l 1 $\times$ EmeraldAmp GT PCR Master Mix (Takara Bio/Clontech Laboratories, Inc., Mountain View, CA), 8 μ l sterile distilled water, 1 μ l of both reverse and forward primers (10 μ M), and 2.5 μ l fungal cDNA or gDNA. PCR products were separated on a 1% agarose gel (Bio-Rad Laboratories Inc.) and stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA) in 1 $\times$ Tris-acetate-EDTA buffer at 100 V for 1 h. Gel images were taken using KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY).

PCR products were purified using PCR Clean-up & Concentrator (Zymo Research, Irvine, CA) and submitted for Sanger sequencing with both forward and reverse primers at Cornell University Sequencing facility in Ithaca, NY, using their Applied BioSystems Automated 3730xl DNA Analyzer. Additional primers *VisdhC*582R and *VisdhD*433R were used to sequence the purified PCR samples to confirm the position of the start codons for the *VisdhC* and *VisdhD* gene, respectively. All sequencing data were analyzed using CLC Main Workbench. Suspected residue sites for mutations conferring SDHI resistance were identified based on homology to mutations found in other pathosystems. The NCBI BLAST function was used to determine percent homology of *VisdhC* and *VisdhD* to those of other fungal species.

Determination of baseline sensitivity to next-generation SDHI fungicides. To determine the concentration at which 50% of conidial germination and mycelial growth was inhibited by each of the SDHI fungicides (EC_{50} value), in vitro inhibition assays were completed. Technical-grade fluxapyroxad (BASF Corporation), pydiflumetofen (Syngenta Crop Protection, Greensboro, NC), and inpyrfluxam (Valent Technical Company, Dublin, CA) were dissolved in acetone to attain the following final nine concentrations through serial dilutions once amended in media: 0 (control), 0.001, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10 μ g ml^{-1} . Baseline EC_{50} determination was also repeated for technical-grade benzovindiflupyr (Syngenta Crop Protection) as an internal control to ensure these new isolates were also representative of isolates used in previous work done by Villani et al. (2016). Each concentration was amended into autoclaved potato dextrose agar (PDA) (Difco Laboratories) that had cooled to $55^{\circ}C$. PDA

was determined to be the most suitable medium for use with technical grade SDHI fungicides for in vitro sensitivity of *V. inaequalis* by Villani et al. (2016). All media were also amended with 50 µg ml⁻¹ streptomycin sulfate and 50 µg ml⁻¹ chloramphenicol to prevent high levels of bacterial contamination (PDA++).

To assess preventive activity of SDHI fungicides, we conducted a modified conidial germination assay (Villani et al. 2016). Conidial solutions of 100 µl collected from 50 baseline isolates were evenly distributed on PDA++ amended with each SDHI fungicide (fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr) at each concentration as well as a control plate (no fungicide added). Plates were incubated at 22°C for 1 week in 12 h light-dark intervals. After the incubation period, five germ tubes or microcolonies from five individual conidia per plate were measured as replicates using SPOT Idea 3.1 with SPOT Imaging Basic software package (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA). Conidial germination assays were performed for each fungicide, which was independently tested for the same 50 baseline isolates. All fungicides were assessed within the timeframe of 3 months in controlled laboratory conditions. In addition to EC₅₀ values for inhibition of conidial germination, EC₅₀ values for mycelial growth on the four SDHI fungicides were also determined to evaluate postinfection activity. Fifty baseline isolates obtained from stored (2013–2015) or fresh (2016–2017) single-spored conidial suspensions (see above) were plated on PDA++ and incubated for 3 weeks at 22°C. Mycelium was transferred using a 3-mm cork-borer in duplicate on each concentration for the four SDHI fungicides. After 3 weeks of incubation at 22°C, the radial diameter was measured for both plugs on each plate.

For inhibition of both conidial germination and mycelial growth, mean percent relative growth was determined. The effective concentration needed for 50% inhibition based on the nonamended control was calculated using a probit analysis with the Statistical Analysis System (SAS) Version 9.4 (Cary, NC) SAS as previously described (Kreis et al. 2016).

Determination of cross-sensitivity to SDHI fungicides. Given the similarity in target site of the SDHI fungicides, cross-sensitivity of *V. inaequalis* to the six SDHI fungicides was determined. Technical grade fluxapyroxad, pydiflumetofen, inpyrfluxam, penthiopyrad (DuPont Crop Protection), fluopyram (Sigma-Aldrich, St. Louis, MO), and benzovindiflupyr were dissolved in acetone to a concentration of 1× and 10× the baseline EC₅₀ values for each fungicide (determined above). Cross-sensitivity was determined for both inhibition of mycelial growth and conidial germination. The discriminatory doses for penthiopyrad and fluopyram used for analysis of cross-sensitivity were based on EC₅₀ values determined in Villani et al. (2016). EC₅₀ values used for penthiopyrad and fluopyram for conidial germination inhibition were 0.08 and 0.176 µg ml⁻¹, respectively, while EC₅₀ values for mycelial growth inhibition were 0.8 and 2.02 µg ml⁻¹, respectively. The concentration of 10× the EC₅₀ value was selected as a discriminatory dose for consistency between different SDHI fungicides. Due to little to no mycelial growth at a

concentration of 10× the EC₅₀ value, mycelial growth cross-sensitivity was repeated at 1× the EC₅₀ value.

Fifty isolates, consisting of either baseline or exposed phenotypes, were used to assess cross-sensitivity for the stage of conidial germination inhibition. Conidial suspensions (100 µl) from each isolate were evenly distributed on PDA++ amended with each of the six different fungicides as well as two non-fungicide-amended control plates. After 1 week, germ tube length or microcolony diameter was measured as described above. Cross-sensitivity was also determined for inhibition of mycelial growth. Fifty isolates of actively growing *V. inaequalis* cultures from above (4 weeks old) were plated on PDA++ amended with each of the different fungicides, including two non-fungicide-amended control plates with a cork borer 5 mm in diameter. After 4 weeks of incubation at 22°C, diameters of radial growth were measured for each isolate on each fungicide. Percent relative growth was calculated for each isolate on all six SDHI fungicides for both mycelial and conidial growth. Pearson Correlations were determined for all pairwise fungicide combinations using the Proc CORR procedure in SAS, Version 9.4.

Multiple fungicide resistance analysis. We hypothesized that target-site mutations may be conferring the higher relative growth observed in isolates exposed to SDHI fungicides when compared with growth of baseline isolates. To determine if high relative growth values (>50%) were associated with mutations in the target site of SDHI fungicides, DNA was extracted from mycelium of two isolates with the highest percent relative growth on SDHI fungicides as described above. Target genes *VisdhB*, *VisdhC*, and *VisdhD* were amplified as described above and as in Villani et al. (2016) (Table 1). PCR products were purified and sequenced in the forward and reverse direction as described above. All returned sequences were analyzed for SNPs in CLC Main Workbench.

Given the potential for an absence of missense mutations in *Visdh* genes, we wished to determine whether high relative growth values for exposed isolates could be due to multifungicide resistance mechanisms, such as overexpression of multidrug efflux pumps (Hahn 2014; Jurick et al. 2017). We hypothesized that if isolates with reduced sensitivity to SDHI fungicide also had high relative growth values when exposed to fungicides from different classes (e.g., FRAC groups), then multifungicide resistance mechanisms may be involved. To assess the possibility of multifungicide resistance in exposed populations, a subset of isolates from the cross-sensitivity experiments (three baseline isolates and nine exposed isolates with relative growth greater than 50% on a minimum of two SDHI fungicides tested) were subjected to relative growth assays on PDA++ amended with technical grade myclobutanil (FRAC group 3) (Sigma-Aldrich, St Louis, MO), dodine (FRAC group U12) (Sigma-Aldrich, St Louis, MO), and trifloxystrobin (FRAC group 11) (Sigma-Aldrich, St Louis, MO). Myclobutanil, dodine, and trifloxystrobin were dissolved in acetone to obtain discriminatory concentrations of 0.1, 0.2, and 0.02 µg ml⁻¹, respectively, as previously described (Frederick et al. 2014; Köller et al. 2004; Villani et al. 2015). Two 3-mm mycelial plugs growing on PDA were transferred to PDA++ amended with each fungicide at the discriminatory dose as well as two non-fungicide-amended

Table 1. Primers used in identification of *VisdhC* and *VisdhD* genes

Primer name	Amplification target	Sequence (5'-3')	Annealing temperature (°C)
VisdhC-106F ^a	VisdhC upstream (Forward)	TTGGCTTTGACTATTCGG	49
VisdhC+200R	VisdhC downstream (Reverse)	ATTGAGGTGTTTGAGATGAC	49
VisdhC1F	VisdhC coding region (Forward)	ATGATGTCCAACAGACTCTCCA	50
VisdhC654R	VisdhC coding region (Reverse)	TCAAATATACATGTGATCGGC	50
VisdhC582R	VisdhC start (Reverse)	GGAATGTGAATGGTAGAG	47 ^b
VisdhD1F	VisdhD coding region (Forward)	ATGGCTCAATTGCTCA	52
VisdhD713R	VisdhD coding region (Reverse)	TTAGCTCGCGAACCTGCCGA	52
VisdhD-139F	VisdhD upstream (Forward)	TGATCATGGGACCTAAG	50
VisdhD695R	VisdhD middle (Reverse)	CCGAATTTATCCAACGATT	50
VisdhD433R	VisdhD start (Reverse)	AAGCGCTCGAATGTCCAGTG	58 ^b

^a Primer names are based on location of 5' end.

^b Indicates primer not used for PCR but for clarification in sequencing, therefore annealing temperature not used.

control plates. Plates were incubated at 22°C for 3 weeks. Radial growth was measured on each plate, once per colony along the largest diameter, and percent relative growth was calculated for each fungicide as described above.

Results

Identification of *VisdhC* and *VisdhD* genes. The *VisdhC* and *VisdhD* genes were cloned from baseline isolate 10-3-14 to identify

potential mutations that may confer future resistance to SDHI fungicides. *VisdhC* and *VisdhD* genes were successfully amplified and sequenced with the primers listed in Table 1 and deposited into GenBank under accession numbers MH484042 and MH484043, respectively.

Amplification of gDNA of the *V. inaequalis sdhC* gene with primer pairs *VisdhC*-106F/*VisdhC*+200R and *VisdhC*1F/*VisdhC*654R revealed fragments of 589 and 706 bp, respectively.

Fig. 1. Distribution of values of effective concentrations by which growth was inhibited by 50% for baseline isolates of *Venturia inaequalis* for the SDHI fungicides fluxapyroxad (A and B), pydiflumetofen (C and D), inpyrfluxam (E and F), and benzovindiflupyr (G and H). Graphs in light gray (A, C, E, and G) indicate EC₅₀ values for conidial germination inhibition, while graphs in dark gray (B, D, F, and H) indicate EC₅₀ values for mycelial inhibition.

To identify exons (coding region) and introns within *VisdhC*, amplification of cDNA was conducted with primer pair VisdhC1F/VisdhC654R and revealed a 558-bp fragment. Alignment of gDNA and cDNA *VisdhC* sequences yielded introns of 49 and 99 bp occurring at nucleotide positions +41 and +175, respectively. With introns, *VisdhC* was determined to be 712 bp in length. The translated ViSDHC protein had 56% similarity to that of *B. cinerea* and 57%

similarity to *Monilinia fructicola*. Residue 144 (histidine) was noted (S 1) due to its proximity and similarity to common mutations found in other phytopathogens that confer resistance to SDHI fungicides, where a histidine residue is replaced with arginine (e.g., H134R) (Avenot et al. 2009).

Amplification of gDNA of the *V. inaequalis sdhD* gene with primer pairs VisdhD-139F/VisdhD713R and VisdhD-139F/VisdhD695R

Fig. 2. Percent mycelium relative growth values of baseline and exposed isolates on PDA++ medium amended with **A**, fluxapyroxad (0.228 µg/ml), **B**, pydiflumetofen (0.062 µg/ml), **C**, inpyrfluxam (0.0291 µg/ml), **D**, benzovindiflupyr (0.057 µg/ml), **E**, penthiopyrad (0.086 µg/ml), and **F**, fluopyram (2.02 µg/ml). Values represent the mean of two colonies for each isolate. Baseline isolates are to the left of the black line (1-21) and exposed isolates are to the right (22-32).

revealed fragment size of 851 and 835 bp, respectively. Coding regions were amplified with primer pair *VisdhD1f/VisdhD713R* and revealed a fragment size of 600 bp. The *VisdhD* gene was 713 bp (with introns) and allowed us to identify two introns of 59 and 54 bp occurring at positions +233 and +310. Residue 130 (aspartic acid) was noted due to its proximity and similarity to common mutations found in other systems that confer resistance to SDHI fungicides, where an aspartic acid is replaced by an arginine. This correlates with the D133R mutation in *A. alternata* (Avenot et al. 2009) and the D132R mutation in *Sclerotinia sclerotiorum* (Avenot and Michailides 2010). The translated *ViSDHD* protein had 57% homology to that of *B. cinerea*.

Baseline sensitivity to fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr. Baseline sensitivity values for *V. inaequalis* were determined for four SDHI fungicides: fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr. Baseline sensitivities were determined for mycelial growth and conidial germination using growth inhibition assays. Discrepancies in isolate numbers between different fungicide or growth stage sensitivity assessment were the result of high contamination levels of yeast and other fungi. For inhibition of conidial germination, the mean EC_{50} value for fluxapyroxad was $0.0284 \mu\text{g ml}^{-1}$ ($n = 30$) with a range from 0.00159 to $0.160 \mu\text{g ml}^{-1}$ (median = $0.0167 \mu\text{g ml}^{-1}$) (Fig. 1A). The mean EC_{50} value for pydiflumetofen was $0.014 \mu\text{g ml}^{-1}$ ($n = 47$) with a range from 0.00043 to $0.0106 \mu\text{g ml}^{-1}$ (median = $0.00281 \mu\text{g ml}^{-1}$) (Fig. 1C). For inpyrfluxam, the mean EC_{50} value was $0.0137 \mu\text{g ml}^{-1}$ ($n = 41$) with a range from 0.00058 to $0.114 \mu\text{g ml}^{-1}$ (median = $0.008 \mu\text{g ml}^{-1}$) (Fig. 1E). Lastly, to ensure this set of baseline isolates performed similarly to those used by Villani et al. (2016), the mean EC_{50} values for mycelial growth inhibition were again determined for benzovindiflupyr. The mean EC_{50} value for benzovindiflupyr was $0.00213 \mu\text{g ml}^{-1}$ ($n = 47$), with a range of 0.00163 to $0.00337 \mu\text{g ml}^{-1}$ (Fig. 1G).

Compared with the EC_{50} values for conidial germination, EC_{50} values for mycelial growth inhibition were always greater for each of the SDHI fungicides. The mean EC_{50} value for fluxapyroxad was $0.228 \mu\text{g ml}^{-1}$ ($n = 22$), with a range from 0.0236 to $0.65 \mu\text{g ml}^{-1}$ (median = $0.184 \mu\text{g ml}^{-1}$). There was a hundred-fold increase from the min to the max EC_{50} (Fig. 1B). The mean EC_{50} value for pydiflumetofen was $0.062 \mu\text{g ml}^{-1}$ ($n = 47$) with a range from 0.000142 to $1.0317 \mu\text{g ml}^{-1}$ (median = $0.076 \mu\text{g ml}^{-1}$) (Fig. 1D). For inpyrfluxam, the mean EC_{50} value was $0.0291 \mu\text{g ml}^{-1}$ ($n = 32$) with a range from 0.00239 to $0.128 \mu\text{g ml}^{-1}$ (median = $0.0181 \mu\text{g ml}^{-1}$) (Fig. 1F). Mean EC_{50} values for benzovindiflupyr were $0.0575 \mu\text{g ml}^{-1}$ ($n = 47$), with a range of 0.000532 to $0.920 \mu\text{g ml}^{-1}$ (Fig. 1H).

Cross-sensitivities to SDHI fungicides. Due to the similarity in mode of action between all SDHI fungicides, cross-sensitivity was also examined to determine correlations in isolate sensitivity to different SDHI fungicides. The correlation coefficients (r) from each pairwise fungicide combination are printed below (Table 2). For inhibition of conidial germination, cross-sensitivity were overall lower, with the highest correlation seen between fluopyram and penthiopyrad ($r = 0.775$, $P < 0.0001$). High correlations also existed between pydiflumetofen and fluopyram ($r = 0.741$, $P < 0.0001$), inpyrfluxam and benzovindiflupyr ($r = 0.715$, $P < 0.0001$), as well as pydiflumetofen and penthiopyrad ($r = 0.707$, $P < 0.0001$)

(Table 2). Among all SDHI fungicides, there was a significant ($P < 0.05$), positive relationship between all active ingredients with varying degrees of strength (Table 2). For inhibition of mycelial growth of *V. inaequalis*, the highest correlations were between benzovindiflupyr and penthiopyrad ($r = 0.950$, $P < 0.0001$), penthiopyrad and pydiflumetofen ($r = 0.907$, $P < 0.0001$), fluxapyroxad and benzovindiflupyr ($r = 0.902$, $P < 0.0001$), and fluopyram and penthiopyrad ($r = 0.905$, $P < 0.0001$) (Table 2). However, varying levels of significant ($P < 0.05$) positive correlations were observed between all six fungicides.

Interestingly, when observing differences in relative growth between baseline and exposed isolates, there was a very dramatic difference in growth patterns. In baseline isolates, growth was more inhibited, with mean relative growth for baseline isolates on SDHI fungicides ranging from 1.1 to 11.96% when compared with PDA control, depending on the SDHI fungicide. Conversely, mean relative growth for exposed isolates on SDHI fungicides ranged from 31.32 to 75.15%, depending on the SDHI active ingredient (Fig. 2). Overall, baseline isolates were much more sensitive to all six SDHI fungicides than the exposed isolates.

Presence of multiple fungicide resistance. No mutations in the protein sequence of the target site (*sdh* genes) were found in any of the exposed isolates with high relative growth as observed in the mycelial growth inhibition assays. Due to the absence of mis-sense mutations in the *sdh* genes, we hypothesized that the observed insensitivity resulted from a multiple fungicide resistance mechanism that could therefore extend to different classes of fungicides. Indeed, isolates with high percent relative growth on SDHI fungicides also had high percent relative growth on media amended with myclobutanil, dodine, and trifloxystrobin, while all baseline isolates were much more sensitive to these fungicides. The mean relative growth for baseline isolates on myclobutanil, dodine, and trifloxystrobin were 11.56, 7.85, and 1.15%, respectively. Conversely, mean relative growth for exposed isolates on myclobutanil, dodine, and trifloxystrobin were 100.38, 57.71, and 63.33%, respectively, when compared with the PDA control (Fig. 3).

Discussion

Increased reliance on SDHI fungicides for management of apple scab will require precise resistance monitoring for populations of *V. inaequalis*. In order to establish a comprehensive SDHI resistance monitoring program, phenotypic and genotypic characterization of several novel SDHI fungicides was completed for *V. inaequalis*. The *VisdhC* and *VisdhD* genes were characterized for a baseline isolate of *V. inaequalis* to determine potential mutation sites that might confer resistance to SDHI fungicides. Furthermore, baseline sensitivities of *V. inaequalis* mycelium and conidial germination for several SDHI fungicides were determined. *V. inaequalis* baseline sensitivities for fluopyram, penthiopyrad, and benzovindiflupyr have been previously determined (Villani et al. 2016), but baseline sensitivities have yet to be determined for fluxapyroxad, pydiflumetofen, and inpyrfluxam. Lastly, cross-sensitivities for all six SDHI fungicides were determined using both baseline and exposed isolates of *V. inaequalis*.

In this study, the *VisdhC* and *VisdhD* genes were successfully cloned and characterized, and, along with the *VisdhB* gene sequence (Villani et al. 2016), will complete the characterization of the SDHI

Table 2. Pairwise correlations of fungicide sensitivities for each of the six SDHI active ingredients

	Fluopyram	Penthiopyrad	Pydiflumetofen	Benzovindiflupyr	Inpyrfluxam	Fluxapyroxad
Fluopyram		0.775 ^a	0.741	0.689	0.680	0.613
Penthiopyrad	0.905		0.709	0.589	0.677	0.600
Pydiflumetofen	0.865	0.907		0.682	0.596	0.565
Benzovindiflupyr	0.863	0.950	0.871		0.715	0.599
Inpyrfluxam	0.861	0.708	0.670	0.884		0.558
Fluxapyroxad	0.846	0.783	0.820	0.907	0.884	

^a Values in the upper right indicate correlation coefficients (r) for cross-sensitivity based on conidial germination for each fungicide combination, while values in the bottom left are correlation coefficients for cross-sensitivity as determined by mycelial growth. All values are statistically significant ($P < 0.0001$).

binding pocket for *V. inaequalis*. The low percent identity observed between the *VisdhC* and *VisdhD* genes and other fungal organisms is not surprising due to the known lack of conservation of the SDH subunits (Cecchini 2003). This lack of conservation underscores the importance of understanding resistance on a genotypic level, as different mutations have been identified occurring in the *sdhB*, *sdhC*, and/or *sdhD* subunit, as found in *A. alternata* (Avenot et al. 2008, 2009). Different SDHI fungicides might be able to differentially control resistant isolates depending on the mutation present. Certainly, some mutations confer full resistance to SDHI fungicides, while others only confer partial resistance (Sierotzki and Scalliet 2013). For example, in *B. cinerea*, the N230I mutation in the SDHB subunit may confer moderate resistance to boscalid, but low level of resistance to bixafen and carboxin (Veloukas et al. 2013). By comparison, the H272R mutation in the same subunit confers moderate resistance to boscalid, fluopyram, fluxapyroxad, but a lower level of resistance to SDHI active ingredients bixafen and carboxin (Veloukas et al. 2013). While we identified no isolates with mutations that resulted in a change in the protein sequence for any of the genes, the *Visdh* gene sequences will allow for development of primers targeting specific mutation sites identified based on homology to other fungal species to aid in future genotypic monitoring when growers experience product failures.

Baseline sensitivity was also determined for SDHI fungicides fluxapyroxad, pydiflumetofen, and inpyrfluxam in addition to previously determined EC₅₀ values for fluopyram, penthiopyrad, and benzovindiflupyr. Sensitivity was determined for both conidial germination and mycelial growth due to activity of these fungicides against both of these life stages (Villani et al. 2016). Of all SDHI fungicides evaluated in this study, benzovindiflupyr had the highest intrinsic activity against conidial germination of *V. inaequalis* followed by inpyrfluxam, pydiflumetofen, and then fluxapyroxad. For mycelial growth inhibition, inpyrfluxam had the highest activity against *V. inaequalis*, followed by benzovindiflupyr, pydiflumetofen, and then fluxapyroxad. Mean EC₅₀ values for fluxapyroxad were eight times greater for mycelial growth than conidial germination. Pydiflumetofen had the most consistent EC₅₀ value between the two stages of growth (mycelial and conidial). Due to high efficacy against both mycelium and conidia observed, it can be expected that these SDHI fungicides will also have good protectant and curative activity against *V. inaequalis* in the field, with higher efficacy when used as a protectant as opposed to when applied postinfection.

The baseline EC₅₀ values of the SDHI fungicides evaluated in the current study fall within the range of EC₅₀ values analyzed by Villani et al. (2016). While little has been published on some of these SDHI

fungicides in other systems, fluxapyroxad and pydiflumetofen EC₅₀ values for *V. inaequalis* are comparable to what was found in *A. alternata* (Avenot et al. 2014; Olaya et al. 2016). Similar to the high level of intrinsic activity we observed, Olaya et al. (2016) also observed that pydiflumetofen had the highest activity against *A. solani* when compared with several other SDHI fungicides. This is the second time benzovindiflupyr EC₅₀ values have been evaluated for *V. inaequalis*. The range observed here overlapped with the EC₅₀ value for benzovindiflupyr, previously determined by Villani et al. (2016), which demonstrates the consistency between the two sets of isolates used. To our knowledge, this is the first study looking at the efficacy of inpyrfluxam against any fungal phytopathogen. We found that this fungicide has a high level of in vitro activity and potential for application for the management of apple scab due to level of activity comparable with SDHI fungicides registered for use against apple scab.

Differences in the level of intrinsic activity for SDHI fungicides could be explained by differences in affinity for the SDHI target site in *V. inaequalis*. Indeed, the level of homology between the *VisdhC* and *VisdhD* genes and those of the other fungal pathogens of perennial fruit trees (i.e., *M. fructicola*, *A. alternata*, and *B. cinerea*) is quite low. Hence, the intrinsic activity of the SDHI fungicides presented here may be different in other ascomycete pathogens as the fungicides bind to the SDH binding pocket a little differently. Alternatively, the level of intrinsic activity of these SDHI fungicides may be similar for other ascomycetes, but it could be that mutations have a differential effect on the development of insensitivity. While pydiflumetofen and inpyrfluxam are SDHI fungicides currently being developed and evaluated for use in commercial apple production, fluxapyroxad, fluopyram, penthiopyrad, and benzovindiflupyr are all currently registered for use on apple in New York at the time of these experiments. Commercial products containing these active ingredients, such as products like Aprovia or Sercadis, should have high efficacy in the field based on high levels of activity measured in vitro.

Benzovindiflupyr, penthiopyrad, fluxapyroxad, and inpyrfluxam all belong to the chemical group pyrazole 4-carboximide, while pydiflumetofen belongs to the chemical group N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides, and fluopyram belongs to the chemical group pyridinyl-ethyl-benzamide (FRAC 2018). Despite the array of chemical groups that are represented within the class of SDHI fungicides, they all have the same target site, which may be problematic for cross-resistance. At this time, resistance to SDHI fungicides has not yet been reported in *V. inaequalis*, and therefore, cross-resistance remains unknown. However, due to the variation in sensitivities to differing chemistries of SDHI fungicides, we determined cross-sensitivity for a collection of baseline and exposed isolates for all six of these active ingredients.

Significant ($P < 0.0001$) and positive correlations were observed between all SDHIs, with varying degrees of strength of correlation depending on the two fungicides in comparison. The occurrence of cross-sensitivity is not surprising due to similar mode of action between all SDHIs. Benzovindiflupyr, penthiopyrad, fluxapyroxad, and inpyrfluxam are from the same SDHI chemical group, while both fluopyram and pydiflumetofen are from two differing chemical groups. Because of chemistry similarity, we would expect that SDHIs belonging to the same chemical group would have a higher level of cross-sensitivity. Surprisingly, this was not always the case. While high correlations were observed between benzovindiflupyr and penthiopyrad ($r = 0.950$) for mycelial inhibition, two chemistries belonging to the group of pyrazole-4-carboxamides, fluxapyroxad and penthiopyrad, also belonged to the same group, but exhibited a much lower correlation ($r = 0.783$). Similar trends were observed between mycelial and conidial stages between inpyrfluxam and pydiflumetofen. We observed a low correlation for mycelial inhibition ($r = 0.670$) between these two fungicides, which is also mirrored for conidial inhibition ($r = 0.599$). Interestingly, a relatively low correlation was seen between benzovindiflupyr and penthiopyrad ($r = 0.589$) for conidial inhibition, regardless of the aforementioned high correlation for mycelial inhibition. Despite being in different chemical groups, penthiopyrad and

Fig. 3. Percent mycelium relative growth values of baseline and exposed isolates on PDA medium amended with myclobutanil (0.1 µg/ml), dodine (0.2 µg/ml), or trifloxystrobin (0.02 µg/ml). Values represent the mean of two colonies for each isolate. Isolates 1 to 3 are baseline, while 4 to 12 are exposed isolates.

fluopyram were highly correlated for both mycelial and conidial inhibition ($r = 0.905$ and 0.775 , respectively). This same pattern between penthiopyrad and fluopyram was observed by Villani et al. (2016).

The observed cross-sensitivity between specific pairs of SDHI fungicides may be indicative of potential cross-resistance we might observe when resistance develops to certain SDHI fungicides in populations of *V. inaequalis*. Hence, careful monitoring of SDHI control failures in commercial apple orchards will be needed to identify populations shifting toward resistance. While these indications of cross-sensitivity present some concern, it should be noted that cross-sensitivity is not always predictive of cross-resistance. Veloukas et al. (2013) found that fluopyram and boscalid provided different levels of control against varying mutations found in *B. cinerea*. This is supported by Mallik et al. (2014), who found that fluopyram controlled boscalid-resistant isolates of *A. solani*. Such an observation may be slightly misleading given that boscalid and fluopyram are in different SDHI chemical groups. Evaluating cross-resistance once SDHI resistance develops in field populations will bring further insights by illustrating how trends in cross-sensitivity between SDHI fungicides is related to trends in cross-resistance. While correlation does not imply causation, knowledge of correlative cross-sensitivity in *V. inaequalis* further emphasizes the importance of chemical rotation even within the class of SDHI fungicides despite the variation in chemical composition that exists.

While it is expected that there would be no cross-resistance between the SDHIs and other fungicide classes such as DMIs and QoIs due to differences in target site (FRAC 2018), Koller and Wilcox (2001) suggest that populations of *V. inaequalis* with resistance to one fungicide are more likely to subsequently develop resistance to unrelated fungicide chemistries. Such genetic plasticity could explain why the exposed isolates had reduced levels of sensitivity to SDHI fungicides when compared with the baseline isolates. Furthermore, when the subset of baseline and exposed isolates was tested for sensitivity to several fungicide classes, similar levels of insensitivity were observed for all fungicide classes, supporting the idea there may be some form of multiple fungicide resistance playing a role in isolate response. In *B. cinerea*, a mutation in the transcription factor, *Mrr1*, is associated with reduced sensitivity to multiple fungicides caused by overexpression of efflux pumps, denoted as MDR1 (multidrug resistance 1) and MDR2 (Hahn 2014; Jurick et al. 2017). To confirm that a similar phenomenon would be happening in *V. inaequalis*, further genomic investigation directed at mutations in *ViMrr1* would be required. In *V. inaequalis*, multiple fungicide resistance has not been associated with any one genetic determinant or fitness costs (Chapman et al. 2011), suggesting sensitive isolates may not be able to outcompete resistant isolates, which could pose new management challenges for modern fungicide use.

Continual monitoring of SDHI sensitivity is vital for the preservation of this important fungicide tool for managing apple scab. With the addition of baseline sequences of the *VisdhC* and *VisdhD* genes as well as baseline sensitivities for all registered SDHI fungicides on apple, it will now be possible to pursue resistance monitoring on both a genotypic and phenotypic level. Knowledge of baseline sensitivities will benefit phenotypic monitoring by looking for shifts in sensitivity away from baseline, and reference sequences with annotations of where common mutation sites are located will allow for a complete assessment of the *sdh* subunit forming the ubiquinone binding pocket. Overall, SDHI fungicides have good activity against both mycelial and conidial growth stages of *V. inaequalis*. Cross-sensitivity between SDHI fungicides occurs to a certain degree depending on the chemical group. However, it is uncertain if the same correlation in cross-sensitivity will be observed once complete resistance arises in field populations. Interestingly, isolates with previous exposure to fungicides seem to have reduced sensitivity to SDHIs, as well as other classes of fungicides, suggesting that multiple fungicide resistance may play a role in future product failures. Hence, continued monitoring of SDHI resistance and multiple

fungicide resistance will be imperative in populations of *V. inaequalis* in the years to come.

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