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Investigating the defoliating-like (DL) VCG2A pathotype of *Verticillium dahliae* through identification and prediction of secreted proteins from genomes of Australian isolates

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Abstract

In Australia, recent investigations have reported the presence of a number of *Verticillium dahliae* pathotypes (VCG1A, VCG2A and VCG6) that cause disease in cotton and agricultural weeds. During these investigations, it was observed that a VCG2A *V. dahliae* displayed greater virulence than previously reported in local and international isolates. Genome sequencing of seven *V. dahliae* isolates was performed using a mixture of short and long reads sequencing technologies. Of these seven isolates, four were identified as D VCG1A, one ND VCG2A, a recently identified Australian VCG6 as well as a virulent VCG2A, classified as "defoliating-like". The secreted protein repertoire from the genomes of these Australian *V. dahliae* isolates was predicted using four separate signal prediction methods. The consensus secreted protein set of the isolates revealed the presence of 20 proteins that were present in all virulent isolates, including the virulent DL VCG2A, and absent in the nonvirulent VCG2A isolates. Ten of these proteins had a functional annotation and were identified as ligninase H8, lipolytic enzyme, laccase, amine oxidase, spherulin-1A, dipeptidyl-peptidase, monooxygenase, dienelactone hydrolase, carboxypeptidase and fibronectin by BLASTP.

KEYWORDS
Australia, cotton, effectors, genomics, *Verticillium dahliae*, virulence

1 | INTRODUCTION

Verticillium dahliae is a highly destructive soilborne fungal plant pathogen capable of infecting over 400 host plant species, including high-value agricultural crops such as cotton, while also being observed to infect agricultural weeds such as Noogoora burr in Australia (Evans, 1971). Verticillium wilt has been monitored in Australian cotton since 1983 with isolates stored in the NSW DPI Plant Pathology and Mycology Herbarium (DAR) reference collection (Chapman et al., 2016). It was originally believed that only one vegetative compatibility group (VCG) was present in Australian cotton, VCG4B; however, discoveries of VCG2A in 2014, VCG1A in 2015 and VCG6 in 2022 have since been made (Bauer et al., 2014; Chapman et al., 2016; Kirkby et al., 2022).

It was first noted in the 1960s, by Schnathorst and Mathre, that *V. dahliae* isolates exhibited differing levels of virulence in cotton and classified them by their ability to either completely defoliate cotton (defoliating, D pathotype) or cause mild wilt and no defoliation (nondefoliating, ND pathotype) (Pérez-Artés et al., 2000; Schnathorst & Mathre, 1966). Pathotypes D and ND can be identified by duplex, nested polymerase

chain reaction (PCR), allowing rapid identification of *V. dahliae* pathotypes in the laboratory, forgoing the need to perform in-planta experiments. In addition to classification of *V. dahliae* isolates by D and ND pathotypes, VCGs are another means of assigning *V. dahliae* to intraspecific groups, whereby VCGs are determined by an isolate's ability to form heterokaryon structures between two nitrogen-nonutilizing (*nit*) mutants. It is through this heterokaryon formation that VCGs of *V. dahliae* are thought to perform genetic exchange and have the potential to share common gene pools. As such, virulence and the D and ND pathotypes are often associated with VCGs on specific hosts, with VCG1A linked with the D pathotype, and VCG2A and VCG4B with the ND pathotype in cotton (Chapman et al., 2016; Jiménez-Díaz et al., 2006).

Interestingly, in Australia, virulence does not seem so clear cut, with the report of a virulent strain of VCG2A and less virulent strains of VCG1A (Dadd-Daigle et al., 2020; Jensen & Redfern, 2017), compared to the commonly observed association of virulent strains with VCG1A and less virulent strains with VCG2A in other parts of the world. Through prior plant studies, our group has found that isolate DAR82597, a defoliating-like (DL) VCG2A isolate, as well as defoliating VCG1A isolates DAR83175, DAR82592, DAR83138 and DAR83143, showed severe disease scores on cotton and led to significantly faster death in cotton compared to DAR83135, a nondefoliating VCG2A (Dadd-Daigle et al., 2022; isolates SS362, SS60, SS61, SS429, SS434 and SS425 therein).

This manuscript aims to identify parallels in potential effectors and virulence factors involved in the virulence of Australian defoliating and DL *V. dahliae* cotton isolates of VCGs 1A and a newly reported VCG6 (Kirkby et al., 2022), in comparison to a ND VCG2A cotton isolate. With the virulence of our DL *V. dahliae* VCG2A isolate reported here in Australia being inconsistent with the virulence of other VCG2A isolates in Australia and around the world, it is important to consider the underlying mechanisms that may be involved. This manuscript extends upon our previous virulence findings through plant studies of isolates DAR82597, DAR83175, DAR82592, DAR83135, DAR83138, DAR83143 and our recently discovered VCG6, DAR85139. In addition, we aim to add seven new *V. dahliae* genomes to the worldwide repertoire of 37 genomes, representing a 19% increase in the current genomic dataset and an increase from only two Australian genomes publicly available.

2 | MATERIALS AND METHODS

2.1 | *V. dahliae* cultures

V. dahliae isolates were collected from 2010 onwards from infected cotton and *Xanthium occidentale* plants in New South Wales, Australia. Isolate cultures were submitted to the NSW DPI Plant Pathology and Mycology Herbarium (DAR) reference collection as reference cultures, with secondary cultures stored in long-term culture collections at the Australian Cotton Research Institute at Narrabri and the Elizabeth Macarthur Agricultural Institute at Menangle. Isolates were chosen for this study that had previously been investigated for their VCG and virulence on cotton (D VCG1A: DAR83175, DAR82592, DAR83138, DAR83143; VCG2A: DAR83135 [D], DAR82597 [DL]) and *X. occidentale* (VCG6: DAR85139) (Chapman et al., 2016; Dadd-Daigle et al., 2020; Table 1). All isolates were found to be highly virulent on their host plant, with the exception of DAR83135, a nondefoliating VCG2A.

2.2 | Genome sequencing and annotation

Genomes for isolates DAR82597, DAR83135 and DAR85139 were sequenced as previously described by Kirkby et al. (2022). Briefly, genomes were sequenced on an Ion Torrent S5 or Miseq V2 2×250 bp and assembled with Spades. Annotation was also performed using Funannotate with ab initio gene predictors trained with *V. dahliae* JR2 RNA-seq data. In addition, isolates DAR83175, DAR82592, DAR83138 and DAR83143 were sequenced on the GridION Release 18.12.4 using minknow-core-gridion 3.1.20. The raw sequencing files were base-called with guppy basecaller (ont-guppy-for-minknow v. 2.0.10). Short read sequencing was then performed with an Illumina MiSeq V2 2×250 bp. Raw ONT reads were assembled using CANU v. 1.8 and polished using Illumina reads and ntEdits. Genomes were checked for completeness using BUSCO and the fungi_odb9 database.

2.3 | Signal peptide prediction

Signal peptide sequence prediction from sequenced and reference *Verticillium* protein sequences was performed using four separate predictor programs, SignalP v. 5.0, Phobius v. 1.01, WolfPsort v. 0.1 and DeepSig v. 1.0, which were run with SP_prediction.py (Webster, 2021) to generate a consensus list of proteins predicted to have a signal peptide. The consensus list was then filtered for transmembrane helices using tmhmm v. 2.0c and Orthogroups determined with Orthofinder v. 2.5.4. Orthofinder was first performed only on Australian isolates to identify putative orthogroups involved in virulence. Orthofinder was also performed on both Australian isolates and reference sequences from NCBI that had protein information (*V. alfalfae* VaMs.102 [SAMN02953725]; *V. dahliae* VDG1 [SAMN14096571]), VdGwydir [SAMN08984993], Getta Getta [SAMN10457093], Vd12158 [SAMN05924275], Vd12161 [SAMN05929042], Vd12253 [SAMN05924795], Vd12251 [SAMN05929039] and 12008 [SAMN05832575]; and *V. longisporum* VL2 ([SAMEA3436396], VL43 [SAMN15856871], VL145c [SAMN15856873], VL32 [SAMN15856870]).

Putative carbohydrate active enzymes (CAZymes) were predicted using dbCAN2 and PFAM and superfamily domains annotated with InterproScan5 v. 5.52–86.0. Orthogroups found to be absent in the nondefoliating VCG2A (DAR83135), but present in defoliating and DL isolates, were investigated for conserved domains with NCBI's Web CD search tool and phylogenetic trees from both whole protein alignments and conserved domain alignments were inferred from MAFFT v. 7.490 aligned protein sequences with FastTree v. 2.1.11 (Figure S1).

TABLE 1 General *Verticillium dahliae* isolate information and statistics.

Isolate	VCG	Size of genome (bp)	Predicted proteins	Predicted secreted proteins	Host	Cotton growing location	Year	Apoplast localization of secreted proteins (%)
DAR83175	1A	35,573,849	8843	574	*Gossypium hirsutum*	Macintyre	2011	53.31
DAR82592	1A	35,800,352	8273	499	*G. hirsutum*	Namoi	2011	52.51
DAR82597	2A	32,539,468	9472	636	*G. hirsutum*	Namoi	2014	54.87
DAR83135	2A	34,373,995	8978	627	*G. hirsutum*	Namoi	2015	56.14
DAR83138	1A	35,905,352	8799	581	*G. hirsutum*	Namoi	2015	54.39
DAR83143	1A	35,561,515	9005	626	*G. hirsutum*	Macintyre	2015	52.40
DAR85139	6	31,982,796	9494	631	*Xanthium occidentale*	Condobolin	2017	54.68

Abbreviation: VCG, vegetative compatibility group.

3 | RESULTS

Secreted peptide prediction was determined in silico by consensus of SignalP, Phobius, WolfPsort and DeepSig with predicted secreted peptides also determined to be absent for transmembrane helices by tmhmm. The number of predicted secreted proteins in the investigated Australian *V. dahliae* isolates ranged from 499 (DAR82592) to 627 (DAR83135) representing 6.0%–6.9% of the predicted proteome, with 52.4%–56.1% of secreted proteins predicted to localize in the apoplast by ApoplastP (Table 1). CAZymes were annotated for about 27.25%–30.31% of these proteins by dbCAN2 for all isolates. Glycoside hydrolases (GH), polysaccharide lyases, carbohydrate-binding modules and carbohydrate esterases were all present in the predicted secretome of each investigated isolate, with GH comprising the largest group of CAZymes, contributing to around half of all proteins with a CAZyme annotation (13.82%–15.49% of all predicted secreted peptides; Figure 1). Glycosyl transferases were present in low proportions (0.2%–0.4%) in isolates DAR83175 and DAR82592, represented by GT1 in both isolates as well as GT8 in DAR83175, and were absent in other isolates.

Small cysteine-rich proteins (SCPs) were also identified from the sets of secreted proteins by filtering for sequences that had a length of <400 amino acid residues and contained an even number of cysteine residues with a count of four or greater. The total count of SCPs for each isolate is shown in Table 2. The percentage of the secretome for each isolate belonging to SCPs ranged from 21.6% in DAR83175 (VCG1A) to 23.9% in DAR85139 (VCG6). Isolates of VCG1A contained the fewest SCPs (n = 109–146) while DAR85139 (VCG6) and DAR82597 (virulent DL VCG2A) contained the highest number of SCPs (n = 151 and n = 149, respectively). Compared to annotated reference sequences from NCBI, the isolates in this study comprised the fewest SCPs (n = 109–151), with reference sequences *V. dahliae* 12253, *V. dahliae* 12008, *V. dahliae* 12251, *V. dahliae* 12158, *V. dahliae* 12161, *V. dahliae* VDG2, *V. dahliae* Getta Getta, *V. dahliae* Gwydir1A3 and *V. dahliae* VDG1 having between 155 SCPs (*V. dahliae* VDG1) and 197 SCPs (*V. dahliae* 12253).

Orthologous groups of the secretome of Australian cotton *V. dahliae* isolates were determined by Orthofinder v. 2.5.4. Orthogroup profiles of secreted proteins were found to cluster based on VCG (Figure 2). Previously, DAR82597 and DAR83135 have both been assigned as VCG2A by a combination of intergenic spacer (IGS) sequencing, D/ND PCR and *nit* mutant testing (Chapman et al., 2016; Dadd-Daigle et al., 2020). All isolates, excluding DAR83135, have also been shown to be virulent on cotton in Australia. Interestingly, 20 orthogroups of secreted proteins (Figure 2) were found to be present in all isolates with the exclusion of DAR83135. These secreted proteins represented orthogroups OG0000006, OG0000007, OG0000070, OG0000085, OG0000131, OG0000313, OG0000326, OG0000337, OG0000345, OG0000346, OG0000363, OG0000382, OG0000395, OG0000401, OG0000438, OG0000463, OG0000498, OG0000503, OG0000506 and OG0000517 (Table S1). Results of BLASTP against the fungi RefSeq protein database identified all orthogroups as having an identity of >90% to proteins in either *V. dahliae* VdLs.17 or *V. nonalfalfae*, with 10/20 of the proteins characterized. Of these 10 proteins, BLASTP identified OG0000085 in DAR82597 as 'ligninase H8' from *V. dahliae* VdLs.17, having 100% ID across all 352 residues of the reference protein. Interproscan also identified a 'peroxidase' PFAM and 'heme-dependent peroxidase' superfamily supporting the BLASTP result. In addition, OG0000363 was identified by BLASTP as a 'lipolytic enzyme' with 99% ID to *V. dahliae* VdLs.17 across all 434 residues and PFAM and superfamily annotations of 'GDSL-like lipase/acylhydrolase family' and 'SGNH hydrolase', respectively. Three orthogroups, OG0000346, OG0000401 and OG0000517, were identified with superfamily annotations as α/β hydrolases. These proteins were identified by BLASTP as a dipeptidyl-peptidase, dienelactone hydrolase and a carboxypeptidase, respectively, matching most closely to proteins in *V. dahliae* VdLs.17 with similarities of 99%, 100% and 99%. OG0000345 was identified as a spherulin-1A from *V. dahliae* with 99% ID over all 235 residues of the reference sequence and classified with an RmlC-like cupin superfamily. A laccase, amine oxidase and mono-oxygenase were also identified through BLASTP for orthogroups OG0000131, OG0000313 and OG0000382 and were mostly closely related to *V. dahliae* sequences with 95%, 100% and 99% IDs, respectively. Lastly, OG0000503 was identified by BLASTP as a 'fibronectin' protein with 100% ID over 434 amino acid residues to *V. dahliae* VdLs.17. However, only a 'pectin lyase-like' superfamily was annotated by Interproscan for this

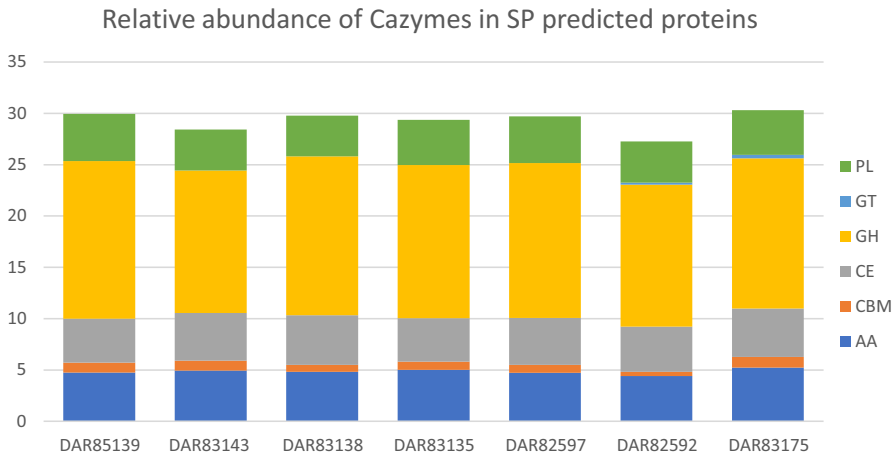

FIGURE 1 Relative abundance of carbohydrate active enzyme annotations of the predicted secretome of Australian *Verticillium dahliae* isolates. SP, signal peptide; PL, polysaccharide lyases; GT, glycosyl transferases; GH, glycoside hydrolases; CE, carbohydrate esterases, CBM, carbohydrate-binding modules; AA, auxiliary activities. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Prediction of secreted proteins and CAZymes.

Verticillium dahliae	DeepSig	SignalP	Phobius	WolfPSort	Consensus	CAZymes	SCPs
DAR82597	899	992	1144	1279	636	189	149
DAR83135	838	917	1050	1185	627	187	135
DAR83138	836	920	1028	1168	581	173	131
DAR83143	905	994	1105	1246	626	178	146
DAR83175	834	917	1045	1178	574	174	124
DAR82592	706	791	904	1061	499	136	109
DAR85139	889	970	1098	1266	631	189	151

Abbreviations: CAZymes, carbohydrate active enzymes; SCP, small cysteine-rich protein.

protein. Of these 20 orthogroups, seven were identified as putative SCPs (OG0000085, OG0000498, OG0000006, OG0000326, OG0000337, OG0000345, OG0000382), with OG0000085, OG0000345 and OG0000382 identified by BLAST as ligninase H8, spherulin-1A and mono-oxygenase, respectively.

Of these orthogroups, 50% (10/20) showed a phylogenetic topology where DAR82597, the DL VCG2A sequenced in this study, the DL reference isolate Getta Getta and the Australian VCG6 isolate grouped together, separate from D VCG1A isolates. These proteins corresponded to orthogroups OG0000085, OG0000007, OG0000498, OG0000337, OG0000313, OG0000401, OG0000363, OG0000346, OG0000463 and OG0000006 (Figure S1; Table S2).

In addition, Orthofinder analysis on the entire proteome of sequenced and reference isolates produced a species tree showing groups of *V. dahliae* based on VCG. Interestingly, the VCG1A group appeared to show a greater variation in branch lengths of isolates compared to other VCG groups, such as 2A and 2B (Figure 3).

4 | DISCUSSION

V. dahliae has a wide host range and variety of pathogenicities linked with VCGs. Historically, VCG1A has been recognized as the pathogenic group of isolates to cause disease in cotton around the world, with VCGs 2A and 4B recognized as less severe than VCG1A. However, in Australia, a highly destructive DL VCG2A pathotype variant discovered causes severe disease in cotton fields, while pathogenic VCG1A frequently displays less virulence than overseas VCG1A counterparts (Dadd-Daigle et al., 2020). The genetic component behind these differences between DL VCG2A and ND VCG2A isolates had not previously been explored until now. Comparative analysis of *V. dahliae* genomes here revealed the presence of 20 predicted secreted proteins to be present in the highly virulent Australian DL VCG2A (DAR82597) as well as Australian VCG1As (DAR83175, DAR82592, DAR83143 and DAR83138), but lacking in the less-virulent nondefoliating VCG2A (DAR83135).

Secreted effectors and virulence factors are essential tools that pathogens require in order to help overcome host defences within the apoplastic zone in plant tissue and cause disease. The differentiation between these pathogenic phenotypes presents clues into the mechanisms that *V. dahliae*, especially in Australia, uses to subvert host defences that continue to cause issues for the Australian cotton-growing industry.

Plants respond to pathogens by recognition of specific pathogen molecular markers, known as pathogen-associated molecular patterns (PAMPs). These markers elicit a PAMP-triggered immunity (PTI) response, the first in the line of defences in plant innate immunity, with PAMPs recognized by host pattern recognition receptors (Zhang & Zhou, 2010). Increased success of pathogens has been linked with mutants in PAMPs in addition to the ability of pathogens to successfully suppress the PTI with secreted

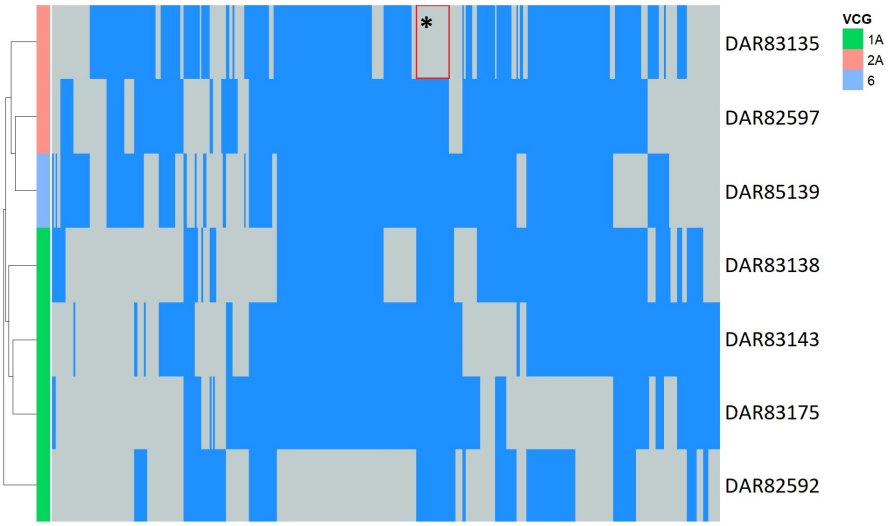

FIGURE 2 Orthologous groups determined by Orthofinder for Australian *Verticillium dahliae* isolates sequenced in this study were clustered using complete-linkage clustering of Euclidean distances. Orthogroups that were found in all isolates were removed so only non-core orthogroups remained. The region marked * represents the absence of 20 secreted protein orthogroups in *V. dahliae* DAR83135 that are present in all other genomes. VCG, vegetative compatibility group. [Colour figure can be viewed at wileyonlinelibrary.com]

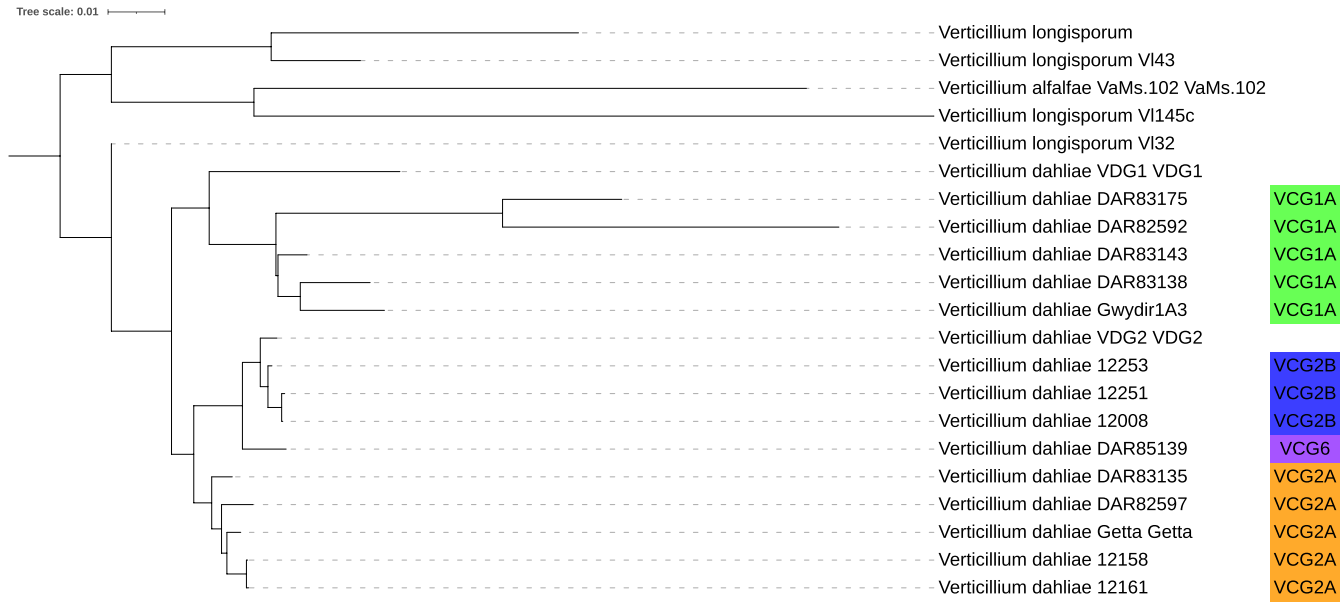

FIGURE 3 Orthofinder species tree of sequenced and reference *Verticillium dahliae* isolates inferred with STAG. VCG, vegetative compatibility group. [Colour figure can be viewed at wileyonlinelibrary.com]

effectors, leading to effector-triggered susceptibility (Koeck et al., 2011; Nicaise & Candresse, 2017). This gamut of secreted effectors is commonly, though not exclusively, defined as protein molecules with an N-terminal secretion signal that directs their secretion via the Golgi apparatus (Lo Presti et al., 2015). To be classed as secreted, the proteins are also devoid of signals that would direct the cell to retain them in other organelles and the cell membrane. Effectors, once secreted, are conveyed to either the plant apoplast or cytoplasm (Rocafort et al., 2020). The apoplast is a harsh intercellular battleground on which plants and their pathogens exchange chemical and enzymatic warfare. This apoplastic space serves as the first barrier between the plant and the environment and as such, pathogens that are well adapted to their host are able to obtain a foothold in these spaces to cause disease (Farvardin et al., 2020; Han et al., 2019).

One secreted protein identified in this study was characterized by BLASTP as a 'ligninase H8' with peroxidase PFAM and Superfamily annotations. Included within the cocktail of compounds in the apoplast, aiding in plant defence, are reactive oxygen species (ROS), involved in the initial oxidative burst in response to pathogen recognition (O'Brien et al., 2012). These ROS are known to play pivotal roles in signalling and responding to biotic and abiotic stresses. Peroxidases play important roles in the conversion of ROS such as diatomic oxygen to the superoxide anion or hydrogen peroxide or the hydroxy radical from hydrogen peroxide and oxygen (Farvardin et al., 2020). Plants themselves use peroxidases as a class of pathogenesis-related proteins (class PR-9) that are induced by pathogen infection (Farvardin et al., 2020). A biphasic oxidative burst response to pathogen infection detailed by an initial low amplitude accumulation of ROS followed by a larger sustained phase has been correlated with disease resistance in

plants (Torres et al., 2006). These peroxidases in plants are also capable of creating physical barriers to inhibit pathogen invasion through the cross-linking of cell wall components (Lamb & Dixon, 1997). In fact, it has been shown that peroxidase knockdown mutants of *Prx33* and *Prx34* in *Arabidopsis thaliana* negatively affected its susceptibility to fungal and bacterial pathogens (Daudi et al., 2012). Pathogen-related peroxidases on the other hand have been shown to be involved in the detoxification of these ROS produced by plants. One such case has been observed in *Magnaporthe oryzae*, in which a deletion mutant of *HYR1*, a glutathione peroxidase, showed significantly smaller lesions on barley and rice in comparison to the wild type and growth inhibition with increased hydrogen peroxide (Huang et al., 2011). The observation of proteins with peroxidase activity and lignin modification in virulent *V. dahliae* pathotypes represents a possible avenue for *V. dahliae* isolates to subvert the host oxidative burst in the initial PTI and/or use the lignin modifying functionality to further promote invasion. This has previously been observed in *V. nonalfafae* with a lignin peroxidase, VnaPRX1.1277, observed in xylem sap in mild and lethal pathotypes infecting hops and with increasing expression during the progression of infection. Knockout lines of these peroxidase genes in *V. nonalfafae* also resulted in an attenuation of virulence, verifying their requirement in colonization (Flajsman et al., 2016). Primarily, the PTI oxidative burst response is made up of hydrogen peroxide (O'Brien et al., 2012) and it is with hydrogen peroxide that lignin can be depolymerized by the hydrogen peroxide-dependent lignin peroxidase (Flajsman et al., 2016). Potential enzymatic use of hydrogen peroxide during the oxidative burst may represent a dual mechanism whereby *V. dahliae* reduces the signalling capability of the initial host oxidative burst response as well as using the oxidative potential of hydrogen peroxide to modify lignin elements. It should be noted that in further analyses of reference *V. dahliae* genomes, two VCG2A *V. dahliae* from strawberry, 12158 and 12161 (GenBank accessions PHNX01000001, PHNU01000001), also contained the same lignin peroxidase orthogroup. These two isolates were observed to be less virulent on their host in comparison to other isolates (Fan et al., 2018). In Fan et al.'s findings, isolates within subclade II-2 (of which 12158 and 12161 were not part of) were shown to have higher virulence on strawberries and contained expanded sets of effector proteins. One of these effector proteins was identified as belonging to the lignin peroxidase family with multiple genes present in the virulent clade as opposed to the single gene present in the non-virulent clade. In addition, *V. dahliae* Getta Getta (GenBank accession CM014041), an Australian cotton VCG2A isolate not sequenced in this study, also showed the presence of this orthogroup and has been confirmed (Linda Smith, Queensland Department of Agriculture and Fisheries, personal communication) to show virulence on Australian cotton, further implicating lignin peroxidase as contributing to the virulent phenotype, at least in Australian DL VCG2A cotton isolates.

In addition, three plant cell wall-degrading enzymes were identified with laccase, lipase and pectate lyase activity in the virulent *V. dahliae* isolates and absent in the nonvirulent VCG2A. Plant-pathogenic fungi secrete lipases to help promote tissue penetration and colonization (Voigt et al., 2005). Inhibition of secreted lipases has been linked with a decrease in virulence previously in both *Botrytis cinerea* and *Alternaria brassicicola* and the role of lipases has been associated with the degradation of lipids and waxes on the plant surface (Mandelc & Javornik, 2015). Among multiple enzyme classes, lipases are a major class of cell wall-degrading enzyme. These cell wall-degrading enzymes have been implicated in important roles for colonization of plant pathogens such as *V. dahliae*, with *V. dahliae*, *V. alfalfae* and *V. nonalfalfae* having been observed to encode more of these enzymes than other plant-pathogenic fungi (Marton et al., 2018). In addition, pectate lyase has also been implicated in the pathogenesis of *V. dahliae* by assisting penetration during infection (Durrands & Cooper, 1988). Pectin is a major component of cell walls in dicotyledonous plants and is an important factor in cell adhesion and cell wall integrity (Xiao & Anderson, 2013). A *V. dahliae*-identified pectate lyase, VdPEL1, was shown to trigger defence responses in both tobacco and cotton plants and similarly, two deletion strains of *VdPEL1* in *V. dahliae* showed significantly reduced virulence on tobacco and cotton plants (Yang et al., 2018). Laccases are a small group of copper-containing proteins that are widely distributed in higher plants and fungi, as well as insects and bacteria (Brijwani et al., 2010). These enzymes are commonly found in white rot fungi and are involved in the degradation of lignin and phenolic compounds (Youn et al., 1995). Laccases have been shown in other fungi to be expressed during pathogenesis and are involved in a number of roles other than virulence. A *LAC1* deletion mutant of *Colletotrichum gloeosporioides* produced less cell wall-degrading enzymes than the wild-type strain (Wei et al., 2017). The authors noted that the deletion mutant was less virulent on wounded and nonwounded mango leaves and fruits, and that the deletion of *LAC1* affected the isolate in terms of mycelial growth and differentiation, conidiation, appressorium formation, pigmentation, melanin biosynthesis, secretion of extracellular hydrolytic enzymes and utilization of exogenous nutrition. The plant cell wall is an important barrier against pathogen invasion of underlying tissue and is composed of heterogeneous components. The difference between the presence of cell wall-degrading enzymes, such as those suggested above, between DAR83135 and DAR82597 provides evidence of their discrepancy in virulence.

Orthogroup OG0000345 was identified by BLASTP to be a 'spherulin-1A' protein, with closest identity to *V. dahliae* VdLs.17 in the Fungal RefSeq database and a superfamily annotation of an 'RmlC-like cupin'. L-rhamnose, with production facilitated by the proteins RmlABCD, has been implicated in virulence of several bacterial pathogens, such as *Salmonella enterica*, *Vibrio cholerae* and *Streptococcus mutans* (Giraud et al., 2000; Santhanam et al., 2017). In fungi, RmlC has been observed in more virulent isolates of *Pyrenophora teres* f. *teres* (Ismail & Able, 2016). In addition, rhamnose synthase activity (RmlD) has been shown to be essential for pathogenicity of *V. dahliae* isolates on tomato and *Nicotiana benthamiana* (Santhanam et al., 2017). In *V. dahliae*, targeted deletion of nucleotide-rhamnose synthase/epimerase-reductase (VdNRS/ER) resulted in the loss of pathogenicity; however, the mutants still were able to grow without any visible negative effects on vegetative growth or sporulation (Santhanam et al., 2017).

Many eukaryotic apoplastic effectors are small proteins of fewer than 400 amino acids and contain multiple, and often even, numbers of cysteine residues. These cysteine residues are theorized to improve stability in the catalytically active environment of the

apoplast (Lo Presti et al., 2015; Stergiopoulos & de Wit, 2009). SCPs accounted for about 21%–24% of all secreted proteins in Australian isolates sequenced in this study. These proteins were putatively described as SCPs having been identified as containing a signal peptide motif, lacking a transmembrane helix, having four or more cysteine residues, being 400 amino acid residues or less in length and resulting in a 'hypothetical' or 'uncharacterized' BLASTP result. Previous reports suggest that *V. dahliae* encodes about 120 of these effector proteins (Wang et al., 2020). Secreted effectors play important roles, contributing to the virulence of phytopathogens, many of which have been identified as small, cysteine-rich proteins (de Jonge et al., 2012; Wang et al., 2020; Zhang et al., 2017). Three SCPs in *V. dahliae*, VdSCP27, VdSCP113 and VdSCP126, were identified to be involved in host–pathogen interactions, eliciting cell death by triggering defence responses, ROS burst, callose deposition and induction of defence genes (Wang et al., 2020). In this study, interestingly VCG1A isolates were observed to produce less of these SCPs than other isolates, including the ND VCG2A isolate. *V. dahliae* VCG1A is associated with the cotton defoliating pathotype and until recently, thought to be exotic to Australia (Chapman et al., 2016; Dadd-Daigle et al., 2020). In addition, the magnitude of damage caused by Australian VCG1A isolates does not appear to parallel international defoliating isolates (Dadd-Daigle et al., 2020). The decrease in SCPs in Australian VCG1A *V. dahliae*, in comparison to VCG2A and VCG6, may be an indication of their lesser impact on cotton, though would require further protein experimentation to verify.

Of the 20 orthogroups observed to be specific to virulent isolates, half ($n = 10$) were observed to show a high similarity between DL VCG2A isolates and the recently identified VCG6 Australian isolate. It is widely accepted that although mating-type genes have been described in *V. dahliae*, there has been no observed sexual stage. As such, genetic exchange is thought to occur through the shared heterokaryon structure formed by two isolates of the same VCG. Previous studies suggest that four main clonal lineages of VCGs exist, VCG1A, VCG1B, VCG2A and VCG4B, with other rarely seen VCGs such as VCG 2B, VCG3 and VCG6 arising through unknown recombination events (Jiménez-Gasco et al., 2014). Perhaps the same mechanism that allowed recombination to occur in VCG6 has also facilitated genetic exchange of virulence genes with Australian VCG2A isolates leading to the DL phenotype observed.

In recent years, there have been multiple discoveries in Australia related to the breadth and diversity of *V. dahliae* isolates in Australian cotton. While it is apparent that there exists a distinction between Australian and overseas pathotypes, the genetic component of these differences is currently not well understood. The results presented here begin to scratch the surface of these differences to better understand how *V. dahliae* in Australia causes disease.

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[Correction added on 28 November 2022, after first online publication: CAUL funding statement has been added.]

DATA AVAILABILITY STATEMENT

Genomes sequenced in this study were submitted to NCBI under BioProject at www.ncbi.nlm.nih.gov/bioproject/ number PRJNA788308. Protein data was downloaded from NCBI for the following isolates/biosamples: *Verticillium longisporum* VL2 (SAMEA3436396), *Verticillium longisporum* VL43 (SAMN15856871), *Verticillium longisporum* VL145c (SAMN15856873), *Verticillium longisporum* VL32 (SAMN15856870), *Verticillium alfalfae* VaMs.102 (SAMN02953725), *Verticillium dahliae* VDG1 (SAMN14096571), *Verticillium dahliae* VdGwydir (SAMN08984993), *Verticillium dahliae* Getta Getta (SAMN10457093), *Verticillium dahliae* Vd12158 (SAMN05924275), *Verticillium dahliae* Vd12161 (SAMN05929042), *Verticillium dahliae* Vd12253 (SAMN05924795), *Verticillium dahliae* Vd12251 (SAMN05929039) and *Verticillium dahliae* 12008 (SAMN05832575).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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