Genomic Profiling of Virulence in the Soybean Cyst Nematode Using Single-Nematode Sequencing

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Accepted for publication 22 October 2020.

ABSTRACT

Soybean cyst nematode (SCN) is one of the most important diseases in soybean. Currently, the main management strategy relies on planting resistant cultivars. However, the overuse of a single resistance source has led to the selection of virulent SCN populations, although the mechanisms by which the nematode overcomes the resistance genes remain unknown. In this study, we used a nematode-adapted single-cell RNA-seq approach to identify SCN genes potentially involved in resistance breakdown in Peking and PI 88788 parental soybean lines. We established for the first time the full transcriptome of single SCN individuals allowing us to identify a list of putative virulence genes against both major SCN resistance sources. Our analysis identified 48 differentially expressed putative effectors (secreted proteins required for infection) alongside 40 effectors showing evidence of novel structural variants, and 11 effector genes containing phenotype-specific sequence polymorphisms. Additionally, a differential expression analysis

revealed an interesting phenomenon of coexpressed gene regions with some containing putative effectors. The selection of virulent SCN individuals on Peking resulted in a profoundly altered transcriptome, especially for genes known to be involved in parasitism. Several sequence polymorphisms were also specific to these virulent nematodes and could potentially play a role in the acquisition of nematode virulence. On the other hand, the transcriptome of virulent individuals on PI 88788 was very similar to avirulent ones with the exception of a few genes, which suggest a distinct virulence strategy to Peking.

Keywords: disease resistance, effector, evolution, genomics, *Heterodera glycines*, host-parasite interactions, nematodes, pathogen effectors, Peking, PI 88788, plant-parasitic nematode, population biology, RNA-seq, single-cell sequencing, virulence

Soybean cyst nematode (SCN, *Heterodera glycines*) is one of the most important diseases of soybean in all major producing countries (Allen et al. 2017; Matsuo et al. 2012; Peng et al. 2016). In the United States and Canada, economic losses caused by SCN have been estimated to be in excess of one billion U.S. dollars each year (Allen et al. 2017). In North America, SCN was first identified in North Carolina in 1954 and has since spread to almost every soybean producing state, reaching Canada in 1987 (Anderson et al. 1988; Mimee et al. 2014; Tylka and Marett 2017; Winstead et al. 1955). The high genetic diversity and continuous gene flow that characterize SCN populations in North America facilitate their adaptation to diverse bioclimatic conditions and consequently their establishment in new areas (Gendron St-Marseille et al. 2018). The ongoing climate warming also favors SCN development by increasing the number of generations per growing season and by opening new regions to soybean cultivation (Gendron St-Marseille

et al. 2019). Therefore, more damages caused by SCN are expected in the future.

Crop rotation and resistant cultivars are still the most effective ways to control SCN (Lund et al. 2018; Wang et al. 2003). Breeding efforts have allowed the development of highly resistant soybean varieties that have remained effective for many decades with little associated yield drawbacks (Rincker et al. 2017; Tylka and Mullaney 2015). However, nearly 95% of these resistant cultivars are derived from a single parental line, PI 88788 (Mitchum 2016; Tylka and Mullaney 2015). In recent years, the overutilization of PI 88788 has exerted a strong selective pressure leading to the emergence of virulent SCN subpopulations able to overcome these resistance genes (McCarville et al. 2017; Niblack et al. 2008; Zheng and Chen 2013). As an example, a recent study showed that PI 88788-type resistance was overcome by SCN populations in all 393 tested fields in Missouri (Howland et al. 2018). Worse yet, in 43% of these fields, SCN populations were found to have the capacity of reproducing on a second source of resistance, PI 548402 (Peking), from which the other 5% of resistant cultivars are derived (McCarville et al. 2017). Thus, it is recommended to implement cultivar rotation in order to limit the development of virulent populations (Niblack 2005). Nevertheless, this situation will remain challenging until the development of new resistant germplasm (Boucher St-Amour et al. 2020) and a sound understanding of how the nematode circumvents mechanisms of soybean resistance is gained.

Soybean resistance is based on the degeneration of the SCN feeding cell, called the syncytium, ending in the necrosis of the host cells around the nematode, a mechanism similar to a hypersensitive response (Mitchum 2016). This phenomenon is polygenic in nature, with many identified quantitative trait loci (QTL) being involved in resistance (Kim et al. 2010). Two major QTLs, *Rhg1* and *Rhg4*, are known major drivers of PI 88788 and Peking resistance (Klink et al.

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Funding: Support was provided by Génome Québec, Genome Canada (Soyagen), the Government of Canada, the Ministère de l'Économie, Science et Innovation du Québec, Semences Prograin Inc., Syngenta Canada Inc., Sevida Genetics, La Coop Fédérée, Grain Farmers of Ontario, Saskatchewan Pulse Growers, Manitoba Pulse & Soybean Growers, the Canadian Field Crop Research Alliance, and Les Producteurs de grains du Québec.

First and second authors contributed equally to this work.

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary tables are published online.

The author(s) declare no conflict of interest.

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2013). Resistance linked to the Peking genotype causes the rapid degeneration of the syncytium, initiated approximately 48 h after infection, and requires the presence of both *Rhg1* and *Rhg4* (Brucker et al. 2005; Liu et al. 2012; Yuan et al. 2012). The resistance associated with PI 88788 takes more time to develop, typically between 8 to 10 days after infection, implicates only *Rhg1*, but requires the presence of multiple copies of the three genes at the *rhg1* locus to be effective (Brucker et al. 2005; Cook et al. 2012, 2014; Liu et al. 2012). The alleles of the α -SNAP (α -soluble N-ethylmaleimide sensitive factor attachment protein) genes present in Peking (*rhg1-a*) and PI 88788 (*rhg1-b*) also differ (Bayless et al. 2016). Although both types of resistance share a similar genetic background, it is generally agreed that SCN populations have evolved independently to each resistance source (Anand and Brar 1983).

Plant endoparasitic nematodes have acquired the capacity to invade their host without triggering natural defense reactions by secreting a diverse set of proteins called effectors (Haegeman et al. 2012). In response, hosts have evolved different resistance mechanisms, typically by recognizing the effectors and initiating a rapid and localized cell death (Williamson and Kumar 2006). With the potato cyst nematode, *Globodera pallida*, the protein Gpa2 produced by the NLR (nucleotide-binding and leucine-rich repeat domains) resistance gene recognizes the *Gp*-Rbp-1 effector protein secreted by the nematode in an incompatible reaction, while a single amino acid polymorphism of this effector gene confers virulence in a compatible reaction (Sacco et al. 2009).

In SCN, many putative effectors have been discovered, but, for most of them, their function remains unknown (Mitchum 2016; Mitchum et al. 2013). Virulence genes were shown to be inherited in a Mendelian mode independently in a dominant (PI 88788) and recessive manner (Peking) using a controlled crossing strategy on inbred SCN lines (Dong and Opperman 1997). Bekal et al. (2015) reported the identification of two candidate virulence genes by comparing highly inbred SCN populations. However, the demonstration of a direct role in virulence and validation in field populations is still needed. Thus, the identity of the genes underlying virulence remains unknown, an indication to their probable complex nature (Mitchum 2016). The recent availability of a reliable genome assembly and annotation of *H. glycines* should facilitate association studies to highlight genes involved in virulence (Masonbrink et al. 2019). Two main hypotheses can be put forward to explain how resistance is overcome. First, a difference in gene expression (resulting from regulation, gene copy number, or epigenetic control for example) could lead to the absence of a protein involved in an effector-triggered immunity (ETI) response or the overproduction of a virulence factor. Second, polymorphism in effector proteins resulting from mutations in the coding sequence, differential exon usage, or alternative splicing could prevent their recognition by the host plant.

To meet the challenges associated with the high genetic diversity of SCN populations (Gendron St-Marseille et al. 2018), and the difficulties of obtaining highly inbred strains to perform genetic analyses, we used a nematode-adapted single-cell RNA-seq protocol to establish the transcriptome of fourth stage (J4) individual females from eight distinct SCN field populations developing on either Peking, PI 88788, or the susceptible cultivar Essex. We describe here the first comparative analysis of the transcriptomic response of SCN to different resistance sources based on single nematode transcriptomic amplification.

MATERIALS AND METHODS

SCN Populations. Eight soybean fields in Ontario, Canada infested by SCN populations with diverse virulence phenotypes were sampled (Table 1). Individual population virulence phenotype (HG type) to both Peking and PI 88788 resistant soybean lines was confirmed using a standard protocol as described in Niblack et al.

(2002) with the susceptible cultivar Essex acting as susceptible control.

Selection of virulent J4 females on resistant genotypes.

Approximately 500 cysts of each SCN population were placed in separate mesh bags and hydrated for 3 weeks in petri dishes filled with tap water maintained at 25°C in an incubator. The water was then changed and hatching second-stage juveniles (J2) were collected over a period of 48 h to standardize nematode age (Audette et al. 2020). Seven-day-old soybean plants (VC staged) were transferred to silicon ice cube trays and inoculated by pipetting ~200 larvae/plant directly onto the roots. A 50:50 mix of wet beach and masonry sand was then added to cover inoculated roots (~2 cm) and plants were maintained in a growth chamber set at 28°C with a 16/8 h light/dark photoperiod. Owing to their very low reproduction rates on both Peking and PI 88788 resistant cultivars, SCN populations ON1, ON21, and ON22 were selected as avirulent controls and were inoculated onto the susceptible soybean line Essex. Selection for avirulent individuals is currently not feasible and, although these populations showed very low female index (FI), there is a possibility that some virulent individuals may develop on Essex. Therefore, the term “avirulent individuals” abbreviated by “Avir” in this paper should be interpreted as individuals from an avirulent population. Nematode populations ON6 and ON16 were inoculated on Peking to select virulent individuals, and ON7, ON17, and ON24 on PI 88788. Twelve plantlets per population were inoculated. After 24 h, the roots were rinsed free of sand and residual J2s before being transferred to plastic cone-tainers (SC10L, Stuewe and Sons, Inc., Tangent, OR, U.S.A.) filled with the same 50:50 mix of sand. Inoculated plants were incubated in a growth chamber under the same temperature and photoperiod conditions for 14 days. After 13 days, nine plants, one per population, were removed and roots were stained with fuchsin as described in Eisenback (2000) to confirm infection and life stage of nematodes. The next day, roots were blended as described by Eisenback (2000) and six individual J4 females were handpicked from each population $\times$ plant genotype combinations, dipped clean in PCR-grade water, and immediately processed for RNA extraction. While some effectors involved in earlier stages of parasitism may have been overlooked, females at stage J4 were selected for this study because the initiation of plant response in incompatible interaction generally occurs after 48 h in Peking and 8 to 10 days in PI 88788. It is reasonable to assume that the genes responsible for virulence must be expressed/repressed from this moment until maturity to avoid recognition and degeneration of the syncytium. Testing 14-day-old J4 females should also greatly reduce the likelihood of selecting avirulent individuals on a resistant plant genotype. Even for PI 88788, the development of avirulent individuals would have stopped at least 4 to 6 days before sampling and small size larvae were discarded during handpicking.

RNA extraction. RNA was extracted from fresh individual females directly after handpicking (14 days after inoculation) using a nematode-adapted Smart Seq 2 method (Serra et al. 2018). Briefly, single females were transferred to a 2- μ l PCR-grade water droplet placed in a thin-walled PCR strip. Using a sterile syringe needle, females were cut before adding 2 μ l of lysis buffer solution containing proteinase K and RNasin ribonuclease inhibitor (Promega). After incubating the samples for 10 min at 65°C and then at 85°C for an additional minute, 1 μ l of both oligo-dT VN primer (10 μ M, 5'-AAGCAGTGGTATCAACGCAGAGTACT30VN-3') and dNTP mix was added. Samples were allowed to incubate at 72°C for 3 min before being transferred to ice. Reverse transcription was then carried out by adding 5.7 μ l of master mix to each tube. This master mix consisted of 0.50 μ l of SuperScriptII reverse transcription enzyme, 0.50 μ l of DTT (100 mM), 0.25 μ l of RNasin ribonuclease inhibitor (40 U μ l⁻¹), 2.00 μ l of Superscript II first-strand buffer (5 $\times$), 2.00 μ l of Betaine (5 M), 0.06 μ l of MgCl₂ (1 M), 0.10 μ l of TSO primer (100 μ M, 5'-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'), and 0.29 μ l of nuclease-free water.

Reverse transcription was carried out in an Agilent PureCycler 8800 thermal cycler preprogrammed as follows: 90 min at 42°C, 14 cycles of 50°C for 2 min and 42°C for 2 min, and 70°C for 15 min. Finally, Phusion high fidelity PCR master mix (Thermo Scientific, Waltham, MA, U.S.A.) was used to amplify cDNA using the IS_PCR primers (100 µM, 5'-AAGCAGTGGTATCAACGCAGAGT-3'). The amplification master mix consisted of 5 µl of HF reaction buffer, 0.75 µl of DMSO, 0.5 µl of dNTP, 0.25 µl of IS_PCR primers, 0.25 µl of Phusion polymerase, 10 µl of cDNA from the reverse transcription step, and 8.25 µl of PCR grade H₂O. Amplification was carried out on the Agilent PureCycler 8800 with an initial denaturation at 98°C for 30 s followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 67°C for 15 s, and elongation at 72°C for 4 min 45 s, and ended by a final elongation at 72°C for 5 min. The quality of the cDNA samples was analyzed with an Agilent DNA 7500 Kit on the Agilent 2100 Bioanalyzer. Three samples per population, presenting low levels of RNA degradation (right-skewed read distribution) along with highest mean read length, were kept for sequencing.

Libraries preparation and sequencing. Sequencing libraries were prepared using the Nextera DNA Flex Library Prep Kit (Illumina) following the manufacturer's guidelines. Library qualities were analyzed using the Agilent 2100 Bioanalyzer system with the Agilent High Sensitivity DNA Kit (Agilent Technologies). Libraries were normalized and sequenced (paired-end 2 × 150 bp) at McGill and Genome Québec Innovation Center (Montréal, Québec, Canada) using one lane on the NovaSeq6000 sequencing platform (Illumina).

Bioinformatic processing. *Differential gene expression analysis.* Using Trimmomatic (v. 0.39) (Bolger et al. 2014), sequences were trimmed to keep those with an average quality score higher than 30 and with a length longer than 50 bp. Abundance of transcripts from single-nematode RNA-Seq samples was determined by alignment to the SCN reference genome (GCA_004148225.1) (Masonbrink et al. 2019) using STAR with the *alignIntronMax* flag set to 75,000 and *quantMode* enabled to GeneCounts (v. 2.7.4a) (Dobin et al. 2012). The edgeR package (v. 3.30.3) (McCarthy et al. 2012; Robinson et al. 2009) was used to normalize read counts and to analyze differential expression between treatments. Individuals were grouped based on their virulence phenotypes and three differential contrasts were evaluated: Peking virulent phenotype versus avirulent phenotype (Pek_{vir} versus Avir); PI 88788 virulent phenotype versus avirulent phenotype (88_{vir} versus Avir); and both virulent phenotypes against each other (Pek_{vir} versus 88_{vir}). A gene was deemed as differentially expressed when the log₂FC was higher than twofold and the adjusted *P* value fell under the 1 × 10⁻⁵ statistical threshold.

A hierarchical clustering of differentially expressed genes (DEG) was performed using R package “stats” (v.4.0) (R Core Team 2019). Clusters were calculated using average of the Euclidean distances between gene expression. A heatmap was generated using the pheatmap package (v.1.0.12) (Kolde 2019). A principal component

analysis (PCA) was conducted using the EdgeR (v. 3.30.3) (McCarthy et al. 2012; Robinson et al. 2009) “plotMDS” function on pseudonormalized counts with the *gene.selection* parameter set to “commun”. PCA visualization was performed using the R package ggplot2 (v.3.3.1) (Wickham 2009). Finally, putative coexpressed regions were identified through sequential clustering of DEG position.

Differential exon usage analysis. Differential exon usage (DEU) between treatments was investigated using the package DEXSeq in R (v. 1.34.0) (Anders et al. 2012). Reads were aligned to the SCN reference genome using the STAR aligner with the *alignMaxIntron* flag set to 75,000. Spliced reads were extracted from coordinate sorted BAM mapping files using scripts provided with the DEXSeq package. The analysis compared the three same contrasts (Pek_{vir} versus Avir, 88_{vir} versus Avir, and Pek_{vir} versus 88_{vir}) using an adjusted *P* value threshold of 1 × 10⁻⁵.

Gene polymorphism analysis. Single nucleotide polymorphism (SNP) and small insertions and deletions (INDELs) were identified using VarScan2 (v.2.4.4) (Koboldt et al. 2012) with parameters *min-coverage* and *min-var-freq*, respectively, set to 10 and 0.20. Candidates were filtered to only retain variants with less than 10% missing data. Variant candidates specific to one phenotype and shared across all individuals present within this phenotype were identified as *condition specific*. These *condition specific* variants were used to identify genes with shared mutations across a virulence phenotype. The impacts of SNPs and INDELs was assessed using SnpEff (v.4.3t) (Cingolani et al. 2012) using default settings with the latest SCN genome and gene annotations supplied (Masonbrink et al. 2019).

In order to identify genes putatively involved in virulence having independently acquired mutations, a gene-wise variant analysis was also conducted. Missense and nonsense variants were identified and mapped against gene locations. Genes containing at least one missense or nonsense variant in all individuals of a virulence phenotype were kept for further analysis.

Gene annotation. Putative signal peptides were identified using the SignalP-5.0 web servers with default Eukaryota settings (Almagro Armenteros et al. 2019). Proteins showing at least 50% probability of containing a signal peptide signature with no transmembrane domains or annotated to known effectors of plant-parasitic nematodes were retained. The longest coding transcript was extracted for each candidate gene and compared with the NCBI nr database (dbV5) using the BLASTp algorithm (v.2.10.0) (Altschul et al. 1997) with a minimum E-value cutoff set to 1 × 10⁻³ to determine putative functions.

RESULTS

Following the inoculation of resistant and susceptible soybean genotypes with SCN, J4 females were isolated from eight populations and from 23 of the 24 samples (one missing data in population ON6). A total of 2,097,520,268 paired-end reads were

TABLE 1. Name, origin, and population virulence phenotype (HG type^a) of soybean cyst nematode (SCN) populations sampled in Ontario, Canada

Name	Location	FI ^b on Peking	FI on PI 88788	HG type	Plant selection ^c
ON1	Harrow	2	4	0	Essex
ON21	Kent	0	8	0	Essex
ON22	NA	1	2	0	Essex
ON6	Bothwell	11	6	1–	Peking
ON16	Amherstburg	29	17	1.2–	Peking
ON7	Rodney	2	26	2–	PI 88788
ON17	Parkhill	5	20	2–	PI 88788
ON24	Dresden	2	24	2–	PI 88788

^a Only on indicator line 1 (Peking) and 2 (PI 88788).

^b FI = female index: mean number of females developing on the resistant line divided by the number on the susceptible control multiplied by 100. Populations with FI > 10 are considered virulent on a given resistance line. In this assay, the number of females on the susceptible check varied from 155 to 405.

^c Plant selection identifies the soybean genotype used to select SCN individuals for subsequent genomic analyses.

obtained from sequencing. The number of paired reads ranged from 64,571,101 to 128,822,060 across the individual samples. Overall, 28,076 of the 29,769 genes present in the *H. glycines* reference genome were detected in this study. A PCA analysis confirmed that gene expression within a treatment (SCN population $\times$ plant genotype) was consistent between replicates (Fig. 1).

Differential gene expression analysis. Contrasting the different virulence phenotype groups revealed 267 DEGs in the Pek_{vir} versus Avir contrast, 19 DEGs in the 88_{vir} versus Avir contrast, and 204 DEGs in the Pek_{vir} versus 88_{vir} contrast. Respectively, 109, 19, and 139 of these genes were upregulated and 158, 0, and 65 were downregulated against their contrast counterparts with many being shared between contrasts (Supplementary Tables S1 to S3).

These DEGs were regrouped into 10 clusters based on their relative abundance (Fig. 2) (Supplementary Tables S1 to S3). Considerable variations in the relative levels of gene expression was found in Pek_{vir} females in contrast to the Avir control (Fig. 2). In the Pek_{vir} individuals, gene clusters four and eight showed considerable upregulation while cluster seven contained many downregulated DEGs. On the other hand, the 88_{vir} females exhibited a similar transcriptome to those developing on the Avir control except for a few upregulated genes, mainly contained in clusters three and nine.

Additionally, the three contrasts between treatments identified 48 DEGs corresponding to putative effector proteins, with 18 containing a signal peptide for secretion and 30 being annotated to known expressed proteins (Table 2). Of particular interest, five of the identified putative effector proteins showed a very consistent

expression profile among all contrasts. Four genes were systematically downregulated in the Pek_{vir} individuals compared with Avir and 88_{vir}. These genes (Hetgly.G000017083, Hetgly.G000017105, Hetgly.G000025902, and Hetgly.G000028833) were coding respectively for a putative gland protein G17G01, a Scavenger receptor cysteine-rich (SRCR), pectin-lyase and lectin-like domains-containing protein, and two dorsal gland proteins. One gene was upregulated in the Pek_{vir} individuals compared with both Avir and 88_{vir} (Hetgly.G000016202) and annotated as a dorsal gland protein. Finally, while no putative effectors were exclusively found in 88_{vir} individuals, only three were upregulated compared with unselected females from Essex. Two of these genes were unannotated (Hetgly.G000023122 and Hetgly.G000023125) while the other coded for a dorsal gland protein (Hetgly.G000025567).

Furthermore, many putative coexpressed regions were also observed through tandemly located differentially expressed genes (Supplementary Tables S1 to S3). The largest of these coexpressed regions was highlighted in the Pek_{vir} versus Avir contrast and spanned 10 upregulated genes starting with Hetgly.G000015124 and finishing with Hetgly.G000015137. Across all contrasts, a total of 65 coexpressed regions were identified spanning between 2 and 10 genes. In the Pek_{vir} versus Avir contrast, 138 DEGs (51.7% of the DEGs) were associated with these putative coexpressed regions. Similarly, 6 (31.6%) and 81 DEGs (39.7%) were identified in putative coexpressed regions in the 88_{vir} versus Avir and Pek_{vir} versus 88_{vir} contrast, respectively.

Differential exon usage analysis. The DEU analysis highlighted 148, 14, and 148 exon features with differential usage

Fig. 1. Principal component (PC) analysis of virulence-wise gene expression in soybean cyst nematode individuals.

across the Pek_{vir} versus Avir, 88_{vir} versus Avir, and Pek_{vir} versus 88_{vir} contrasts, respectively (Supplementary Tables S4 to S6). Additionally, 40 DEUs were identified in genes annotated to known effectors ($n = 16$) or containing a signal peptide for secretion ($n = 24$) (Table 3). Six of these DEUs, identified in Pek_{vir} females, showed consistent fold change values across all contrasts. These included three cases of exon upregulation in both Pek_{vir} versus Avir and Pek_{vir} versus 88_{vir} contrast in genes annotated to a CRE-LET-268 protein (Hetgly.G000004724) and two dorsal gland cell-specific expressed proteins (Hetgly.G000016183 and Hetgly.G000016208). Similarly, three cases of exon downregulation in unannotated genes were also identified: two within Hetgly.G000005233 and one within Hetgly.G000013629. Across the 213 unique genes identified as containing DEUs, 199 had only one annotated transcript within the reference genome. One such example was observed in the single transcript gene Hetgly.G000006578. The DEU analysis on this gene suggests a Pek_{vir} specific variant in which exon number six is likely skipped entirely resulting in a seven rather than an eight exon transcript (Fig. 3).

Gene-wise polymorphism analysis. The SNP and INDEL analysis identified 234,620 variants with 198,726 SNPs and 35,865 indels. Of these, 49 heterozygote and 97 homozygote variants were found to be condition specific to the Pek_{vir} phenotype (Supplementary Table S7) with 22 leading to a missense impact on the resulting proteins, and affecting 16 unique genes (Table 4). No such variants were identified in the 88_{vir} individuals or in the Avir control.

A broader gene-wise analysis, aimed at identifying independently acquired missense and nonsense variants in common putative virulence genes, highlighted an additional 23 Pek_{vir} specific genes. In these candidates, five were characterized by a single variant differing only in zygosity (with some individuals being homozygous and some heterozygous). The number of genes impacted by missense and nonsense variants in all individuals was lower in 88_{vir} ($n = 14$) (Table 4). These 52 unique genes contained 460 variants, with 420 missense (91,3%) and 40 nonsense impacts. Additionally, within the genes identified through this gene-wise polymorphism analysis, 11 were putative effector proteins with seven containing a signal peptide for secretion and four having annotations to known effectors of plant-parasitic nematodes (Table 4).

DISCUSSION

We sequenced the whole transcriptome of single SCN females developing on resistant or susceptible soybean lines. Until recently, sequencing the whole transcriptome (or genome) of individual nematodes was impossible due to the small size of these animals and their low nucleic acid content (Serra et al. 2018). While some earlier “single-cell” protocols could result in partial representation of the transcriptome, the technology has evolved rapidly and now allows high-resolution transcriptomic analysis of individual cells (Kolodziejczyk et al. 2015). To our knowledge, this is the first use of a “single-cell” RNA sequencing approach to study gene expression in single individuals of plant-parasitic nematodes. Here, our sequencing, using a nematode-adapted single-cell RNA-seq protocol, resulted in a 94.3% coverage (28,076 out of 29,769 genes) of the predicted transcripts in the *H. glycines* transcriptome (Masonbrink et al. 2019). For the first time, this nematode-adapted single-cell method, initially developed using *C. elegans* (Serra et al. 2018, Chang et al. 2021), allows the exploration of gene expression associated with virulence at the individual level in plant-parasitic nematode. With effector proteins being the key to successful parasitism in many plant-parasitic nematode (Juvalé and Baum 2018; Vieira and Gleason 2019), this study focused on the potential involvement of such proteins toward the acquisition of virulence in SCN.

Differential expression of putative effectors. In plant–pathogen interactions, gene expression may contribute to virulence by either

repressing ETI-inducing proteins or overexpressing “virulence factor” proteins (Sonah et al. 2016). Our analysis revealed a great number of differentially expressed genes on Peking while only a few genes were impacted by the selection on PI 88788. Also, the genes that were differentially expressed on both genotypes showed a lower fold change on PI 88788.

In females developing on Peking, gene functions associated with DEGs were diverse. These included genes known to be involved

Fig. 2. Heatmap of differentially expressed genes identified in soybean cyst nematode individuals virulent on Peking or PI 88788 compared with avirulent, clustered based on their Euclidean distances.

in posttranslational proteins modifications, through kinase and ubiquitin functions, with some involved in DNA replication and transcription and many proteins with putative transporter functions among others. Alongside this diversity in gene functions associated with DEGs, global gene expression was also considerably varied. This could be explained by the fact that the ETI response associated with resistance mechanisms in Peking roots is not only faster but also more aggressive than in PI 88788, forcing the nematode to quickly and aggressively counteract plant defensive mechanisms (Gillet et al. 2017; Mitchum 2016). As shown, plant genotype exerts

a strong selective pressure that is not solely limited to virulence genes. The internal conditions of plant cells from these different resistance genotypes could also differ significantly, explaining these extended changes in gene expression on Peking.

Interestingly, a large proportion of putative effector proteins were upregulated or downregulated in Peking virulent individuals. Because virulence genes are the usual targets associated with the initiation of ETI in the host, their expression could hinder survivability (Masonbrink et al. 2019). Thus, finding effectors exhibiting reduced gene expression is likely. Of the five effectors

TABLE 2. Differentially expressed putative effector genes in soybean cyst nematode virulent individuals

Gene ID	Protein annotation	SignalP score ^a	Contrast groups ^b (Log ₂ FC) ^c		
			Pek _{vir} versus Avir	88 _{vir} versus Avir	Pek _{vir} versus 88 _{vir}
Hetgly.G000002019	CLAVATA3/ESR (CLE)-related protein 2 (Hg-G4G12)	0.922	-4.987	—	—
Hetgly.G000002907	Dorsal gland cell-specific expression protein	—	3.593	—	—
Hetgly.G000004176	NA	0.731	5.549	—	—
Hetgly.G000006633	Papain family cysteine protease	0.968	2.971	—	—
Hetgly.G000009834	Digestive organ expansion factor homolog	0.977	-2.591	—	—
Hetgly.G000010820	Dorsal gland cell-specific expression protein	—	—	—	3.416
Hetgly.G000010858	Dorsal gland cell-specific expression protein	—	—	—	3.070
Hetgly.G000011282	Probable ATP-dependent RNA helicase DHX35 homolog	0.848	4.077	—	—
Hetgly.G000012903	Dorsal gland cell-specific expression protein	—	-4.347	—	—
Hetgly.G000013676	Dorsal gland cell-specific expression protein	—	6.920	—	—
Hetgly.G000015389	Dorsal gland cell-specific expression protein	—	—	—	7.260
Hetgly.G000015914	Dorsal gland cell-specific expression protein	—	—	—	-3.097
Hetgly.G000016165	Dorsal gland cell-specific expression protein	—	2.278	—	—
Hetgly.G000016166	Dorsal gland cell-specific expression protein	—	—	—	4.528
Hetgly.G000016175	Dorsal gland cell-specific expression protein	—	—	—	5.098
Hetgly.G000016202	Dorsal gland cell-specific expression protein	—	3.225	—	3.751
Hetgly.G000016219	Dorsal gland cell-specific expression protein	—	—	—	2.784
Hetgly.G000016226	Dorsal gland cell-specific expression protein	—	—	—	4.875
Hetgly.G000016417	NA	0.978	—	—	4.352
Hetgly.G000017083	Putative gland protein G17G01	—	-5.198	—	-5.386
Hetgly.G000017103	NA	0.945	—	—	-4.055
Hetgly.G000017105	SRCR domain and parallel beta-helix repeat and pectin lyase fold/virulence factor domain and pectin lyase fold domain and C-type lectin-like domain and C-type lectin fold domain and SRCR-like domain-containing protein	—	-2.983	—	-2.681
Hetgly.G000017194	Papilin (abnormal cell migration protein 6)	0.933	—	—	-3.566
Hetgly.G000018654	Dorsal gland cell-specific expression protein	—	-4.295	—	—
Hetgly.G000020834	Transthyretin-like protein 5	0.963	—	—	3.585
Hetgly.G000020893	NA	0.623	-2.229	—	—
Hetgly.G000021247	Dorsal gland cell-specific expression protein	—	-3.168	—	—
Hetgly.G000021325	NA	0.972	-2.719	—	—
Hetgly.G000021339	Mast cell protease 1 (EC 3.4.21.-)	0.988	2.511	—	—
Hetgly.G000021417	Dorsal gland cell-specific expression protein	—	-3.299	—	—
Hetgly.G000023122	NA	0.980	—	6.618	—
Hetgly.G000023125	NA	0.723	—	7.715	—
Hetgly.G000024469	Dorsal gland cell-specific expression protein	—	2.133	—	—
Hetgly.G000024563	Dorsal gland cell-specific expression protein	—	—	—	-8.352
Hetgly.G000025038	NA	0.986	-5.207	—	—
Hetgly.G000025040	Secreted SPRY domain-containing protein 4	—	-5.125	—	—
Hetgly.G000025410	NA	0.987	—	—	2.972
Hetgly.G000025567	Dorsal gland cell-specific expression protein	—	—	5.928	—
Hetgly.G000025628	Putative esophageal gland cell protein Hgg-20	—	-3.790	—	—
Hetgly.G000025902	Dorsal gland cell-specific expression protein	—	-2.766	—	-2.376
Hetgly.G000026741	NA	0.980	-4.204	—	—
Hetgly.G000027281	Putative effector protein	—	—	—	-8.186
Hetgly.G000028281	Dorsal gland cell-specific expression protein	—	—	—	2.923
Hetgly.G000028515	NA	0.988	—	—	-7.455
Hetgly.G000028638	Dorsal gland cell-specific expression protein	—	-4.689	—	—
Hetgly.G000028821	Dorsal gland cell-specific expression protein	—	-3.474	—	—
Hetgly.G000028833	Dorsal gland cell-specific expression protein	—	-5.562	—	-5.229
Hetgly.G000029269	Dorsal gland cell-specific expression protein	—	—	—	-3.128

^a SignalP score predicts the presence of secretory signal peptides.

^b Contrast groups: Peking virulent individuals versus avirulent individuals (Pek_{vir} versus Avir), PI 88788-virulent versus avirulent (88_{vir} versus Avir), and Peking-virulent versus PI 88788-virulent (Pek_{vir} versus 88_{vir}).

^c Log₂FC: Log₂ fold change of expression values.

identified as consistently expressed in all contrasts in Peking-virulent females, all but one (Hetgly.G000016202) were downregulated. This pattern of effector downregulation extended to most effector candidates identified when comparing females developing on Peking to avirulent females (17 downregulated and 9 upregulated). This multilocus change in gene expression might indicate that virulence to Peking has not been acquired in a *gene-for-gene* relation, but rather in a polygenic manner. This would be consistent with previous works highlighting the likely polygenic nature of virulence on Peking (Dong and Opperman 1997; Gardner et al. 2017). Nevertheless, we cannot discount the possibility that a single virulence gene (up- or downregulated) might affect expression of other genes through metabolic changes thus complicating identification. Among these differentially expressed effectors, we identified a CLAVATA3/ESR (CLE)-related protein. This class of secreted plant peptide mimicking effectors are likely involved in the developmental reprogramming of root cells to form the syncytium (Lu et al. 2009). Interestingly, it was reported that soybean hosts were able to specifically recognize CLE effectors containing sequence variants within their variable domains (Mitchum et al. 2012; Wang et al. 2010).

In contrast, putative effector gene expression in females developing on PI 88788 was consistently upregulated with the top three most differentially expressed genes being putative effectors. These included two unannotated genes containing signal peptides for secretion (Hetgly.G000023122 and Hetgly.G000023125), identified as part of a greater cluster of five tandemly located differentially expressed genes, and a dorsal gland cell-specific expression protein (Hetgly.G000025567). In PI 88788, resistance is partially mediated through the progressive accumulation of *rhg1-b* allele with more gene copies resulting in a stronger resistant response to SCN (Cook et al. 2012; Mitchum 2016). Having coevolved together, it is thought that the negative selection pressure applied by virulent nematodes might have selected for this increase (Lee et al. 2015; Yan and Baidoo 2018). From this perpetual arms race, it stands to reason that virulence in SCN might have arisen in a similar fashion through evolutionary increases in expression of the original effector gene. With this in mind, finding one or a few upregulated genes, either by increases in genomic copy number or promoter mutation, is not surprising. In fact, this *gene-for-gene* evolutionary pathway leading to the overexpression of a virulence

TABLE 3. Putative effector genes with differentially used exons in virulent soybean cyst nematode individuals

Gene ID	Feature ID	Protein annotation	SignalP score ^a	Contrast groups ^b (Log ₂ FC) ^c		
				Pek _{vir} versus Avir	88 _{vir} versus Avir	Pek _{vir} versus 88 _{vir}
Hetgly.G000022323	E006	PAN domain containing protein	0.719	1.884	–	–
Hetgly.G000020016	E002	NA	0.802	–3.273	–	–
Hetgly.G000020174	E001	NA	0.824	–	–	2.209
Hetgly.G000007148	E005	Onchocystatin	0.832	–	–	–2.236
Hetgly.G000026395	E002	Hypothetical protein CAEBREN_14776	0.854	2.277	–	–
Hetgly.G000019060	E001	Hypothetical protein GCK72_003741	0.860	–	–	–15.671
Hetgly.G000019060	E002	Hypothetical protein GCK72_003741	0.860	–	–	0.402
Hetgly.G000004724	E001	CRE-LET-268 protein	0.866	2.494	–	2.554
Hetgly.G000020786	E008	FAD-binding protein	0.892	2.815	–	–
Hetgly.G000026104	E006	Glutathione S-transferase-1	0.898	1.627	–	–
Hetgly.G000013629	E006	NA	0.899	–1.929	–	–1.611
Hetgly.G000025030	E009	Guanylyl cyclase	0.934	–	–	3.008
Hetgly.G000004195	E003	Hypothetical protein FO519_009668, partial	0.939	2.330	–	–
Hetgly.G000017719	E013	Hypothetical protein L596_001708	0.950	–	–	–3.448
Hetgly.G000021312	E004	Neutral trehalase	0.950	2.960	–	–
Hetgly.G000021312	E005	Neutral trehalase	0.950	2.734	–	–
Hetgly.G000021312	E016	Neutral trehalase	0.950	–4.817	–	–
Hetgly.G000009291	E004	NA	0.953	–	–	–2.466
Hetgly.G000005248	E008	NA	0.960	4.468	–	–
Hetgly.G000006510	E015	Hypothetical protein FO519_003113	0.972	–1.505	–	–
Hetgly.G000005233	E002	NA	0.986	–5.382	–	–4.882
Hetgly.G000005233	E003	NA	0.986	–2.993	–	–2.440
Hetgly.G000005233	E005	NA	0.986	2.252	–	–
Hetgly.G000007174	E011	NA	0.997	–	–	2.366
Hetgly.G000005243	E003	AChain A, Glutathione synthetase-like effector 22 (Gpa-GSS22-closed)	–	1.650	–	–
Hetgly.G000006576	E002	Pollen-specific leucine-rich repeat extensin-like protein 2	–	2.561	–	–
Hetgly.G000013101	E004	Dorsal gland cell-specific expression protein	–	–	3.072	–
Hetgly.G000013639	E003	Putative gland protein G17G01	–	–4.321	–	–
Hetgly.G000016183	E009	Dorsal gland cell-specific expression protein	–	5.592	–	5.024
Hetgly.G000016183	E012	Dorsal gland cell-specific expression protein	–	15.395	–	–
Hetgly.G000016201	E005	Dorsal gland cell-specific expression protein	–	–	–	–3.377
Hetgly.G000016208	E004	Dorsal gland cell-specific expression protein	–	15.960	–	16.151
Hetgly.G000016219	E004	Dorsal gland cell-specific expression protein	–	–	–	–3.787
Hetgly.G000017094	E009	Scavenger receptor cysteine-rich domain protein	–	1.931	–	–
Hetgly.G000017094	E003	Scavenger receptor cysteine-rich domain protein	–	–	–	–2.439
Hetgly.G000021237	E004	Dorsal gland cell-specific expression protein	–	2.584	–	–
Hetgly.G000022056	E004	Dorsal gland cell-specific expression protein	–	2.045	–	–
Hetgly.G000024333	E002	Dorsal gland cell-specific expression protein	–	–	–	0.810
Hetgly.G000024471	E010	Dorsal gland cell-specific expression protein	–	–4.963	–	–
Hetgly.G000028712	E004	Dorsal gland cell-specific expression protein	–	–	–	2.030

^a SignalP score predicts the presence of secretory signal peptides.

^b Contrast groups: Peking virulent individuals versus avirulent individuals (Pek_{vir} versus Avir), PI 88788-virulent versus avirulent (88_{vir} versus Avir), and Peking-virulent versus PI 88788-virulent (Pek_{vir} versus 88_{vir}).

^c Log₂FC: Log₂ fold change of expression values.

factor has been shown in some plant-parasitic nematode (Castagnone-Sereno 2002).

Still, the very limited difference in gene expression between the individuals developing on PI 88788 and Essex is unexpected. There is a very unlikely possibility that a virulent individual was picked from Essex (chance under 2% according to FI), which would greatly reduce the chances of finding significant differences. Also, our study focused on the J4 stage and while it is reasonable to hypothesize that the expression of the genes involved in virulence would need to be modified from syncytium establishment through nematode maturity, it is also possible that some genes were missed in J4 if their expression had been modified only in earlier stages. Also, because the plant response is slower in PI88788, it could be possible that avirulent individual did develop to the J4 stage. In that case, because the FI of these populations was around 25% on PI 88788, most of the developing females would be avirulent, thus greatly confounding the results. Although very unlikely, we cannot exclude such a pattern.

In a similar way, the major differences in gene expression observed on Peking are probably not all directly related to virulence. As already mentioned, the conditions prevailing in that specific plant genotype probably influenced gene expression. A

developmental delay was also reported in virulent nematode (Li et al. 2004), which could influence the comparison of gene expression after 14 days on resistant versus susceptible lines when individuals are not at the same developmental stage.

Using an allelic imbalance analysis on SCN populations segregating from a virulent (TN10) and an avirulent (TN20) inbred strains, Bekal et al. (2015) highlighted a putative virulence gene named *HgSLP-1* and suggested a direct interaction with the α -SNAP protein encoded by *Rhg1*. They revealed a major drop in *HgSLP-1* copy number in virulent populations and suggested that the absence of this protein could promote virulence. This gene was also identified in our study but the expression across individual replicates as well as across virulence phenotypes varied considerably and no association with virulence could be established.

The high occurrence of coexpressed tandemly located genes in our data were also surprising and may be evidence of common multigene promoters. In response to genotype specific stimuli, a common upstream promoter may be activated resulting in the simultaneous rapid expression of multiple genes (Abad et al. 2008; Pettitt et al. 2014). A similar mechanism is hypothesized in the nematode *G. rostochiensis* through the presence of an upstream dorsal gland master regulator coordinating the

Fig. 3. Schematic view of generalized linear model-derived expression of exons for Hetgly.G000006578 in the avirulent and Peking-virulent (HG1) individuals. Exons showing significant differential usage are in pink. The black transcript indicates the current transcript model found in the reference transcriptome.

expression of effectors (Eves-van den Akker et al. 2016; Kikuchi et al. 2017).

Prevalence of effector sequence polymorphisms. Effector diversification through sequence polymorphisms may also play a key role in the acquisition of virulence, by rendering ETI-inducing genes unrecognizable to the host or by neofunctionalization of these genes (Lilley et al. 2018; Na and Gijzen 2016). Our findings indicate that differential exon usage, a proxy to alternative isoform

regulation (Anders et al. 2012), is much more prevalent in females developing on Peking than those on PI 88788 when compared with their unselected avirulent counterparts. Again, this could potentially be explained by the greater complexity of resistant mechanisms in Peking cultivars compared with PI 88788; through the additional requirement of *Rhg4*. Also, the proportion of DEUs identified in putative effectors was considerable in Peking-virulent females and included known effector genes, such as a glutathione

TABLE 4. Genes containing missense or nonsense variants in virulent soybean cyst nematode individuals

Gene ID ^a	Protein annotation	SignalP score ^b	Variants (n)			
			Total	Missense	Nonsense	vir ^c
Hetgly.G000021835	Mucin-19	0.564	27	26	1	88 _{vir}
Hetgly.G000020013	NA	0.597	2	2	0	Pek _{vir}
Hetgly.G000007807	NA	0.769	2	2	0	Pek _{vir}
Hetgly.G000011601	Hypothetical protein F2P81_025385	0.837	13	13	0	Pek _{vir}
Hetgly.G000009820	NA	0.955	7	6	1	88 _{vir}
Hetgly.G000006510	Hypothetical protein FO519_003113	0.972	10	9	1	Pek _{vir}
Hetgly.G000018368	NA	0.984	8	8	0	Pek _{vir}
Hetgly.G000004693	Maltase-glucoamylase, intestinal	0.997 (T)	2	2	0	Pek _{vir}
Hetgly.G000027197	Maltase-glucoamylase, intestinal	0.997 (T)	1	1	0	Pek _{vir}
Hetgly.G000001227	ATP-dependent DNA helicase PIF1	–	6	6	0	Pek _{vir}
Hetgly.G000003011	Elongation factor 1-beta	–	1	1	0	Pek _{vir}
Hetgly.G000003891	Ribosome-binding protein 1	–	31	29	2	88 _{vir}
Hetgly.G000004483 (*)	Pollen-specific leucine-rich repeat extensin-like protein 2	–	11	10	1	88 _{vir}
Hetgly.G000006545	NA	–	1	1	0	Pek _{vir}
Hetgly.G000007387	Leucine Rich Repeat family protein	–	1	1	0	Pek _{vir}
Hetgly.G000009280	Galactose-1-phosphate uridylyltransferase	–	5	5	0	Pek _{vir}
Hetgly.G000009293	NA	–	1	1	0	Pek _{vir}
Hetgly.G000009298	Unnamed protein product	–	7	6	1	Pek _{vir}
Hetgly.G000009569	Protein lin-25	–	2	2	0	Pek _{vir}
Hetgly.G000009821	NA	–	18	14	4	Pek _{vir}
Hetgly.G000010066	Pao retrotransposon peptidase family protein	–	50	48	2	88 _{vir}
Hetgly.G000010088	TPA_asm: integrase	–	22	19	3	88 _{vir}
Hetgly.G000010093	TPA_asm: PolB-C	–	10	9	1	88 _{vir}
Hetgly.G000010364	Sodium-dependent phosphate transport protein 1	–	1	1	0	Pek _{vir}
Hetgly.G000011645	DAD family protein	–	2	2	0	Pek _{vir}
Hetgly.G000013439	Unnamed protein product	–	5	4	1	Pek _{vir}
Hetgly.G000015085	NA	–	7	5	2	Pek _{vir}
Hetgly.G000016785	Zinc metalloproteinase nas-36	–	4	4	0	Pek _{vir}
Hetgly.G000017094 (*)	Scavenger receptor cysteine-rich domain protein	–	3	3	0	Pek _{vir}
Hetgly.G000017147	NA	–	6	5	1	88 _{vir}
Hetgly.G000017742	DNA topoisomerase	–	2	2	0	Pek _{vir}
Hetgly.G000018116	E3 ubiquitin-protein ligase UBR5	–	4	3	1	Pek _{vir}
Hetgly.G000018273	Putative uncharacterized transposon-derived protein F54H12.3	–	5	3	2	88 _{vir}
Hetgly.G000018276	Unnamed protein product	–	28	26	2	88 _{vir}
Hetgly.G000018620	Unnamed protein product	–	3	3	0	Pek _{vir}
Hetgly.G000018637	Serine threonine protein kinase-related domain containing protein	–	1	1	0	Pek _{vir}
Hetgly.G000018640	Collagen alpha-1(XV) chain	–	9	9	0	Pek _{vir}
Hetgly.G000018643	Hypothetical protein L596_018892	–	2	2	0	Pek _{vir}
Hetgly.G000021548	NA	–	2	2	0	Pek _{vir}
Hetgly.G000021578 (*)	Putative esophageal gland cell protein Hgg-20	–	2	2	0	Pek _{vir}
Hetgly.G000021580	Hypothetical protein LOAG_00804	–	1	1	0	Pek _{vir}
Hetgly.G000021582	Hypothetical protein L596_018892	–	5	5	0	Pek _{vir}
Hetgly.G000021594	Prolyl oligopeptidase	–	2	2	0	Pek _{vir}
Hetgly.G000022839	DUF659 domain-containing protein	–	7/58	6/53	1/5	Pek _{vir} /88 _{vir}
Hetgly.G000025780	Peptidase M12A domain containing protein	–	3	3	0	Pek _{vir}
Hetgly.G000026679	Unnamed protein product	–	3	2	1	Pek _{vir}
Hetgly.G000027114 (*)	Dorsal gland cell-specific expression protein	–	1	1	0	Pek _{vir}
Hetgly.G000027222	Hypothetical protein DRQ56_04470	–	7	7	0	88 _{vir}
Hetgly.G000027596	Hypothetical protein FO519_000976	–	11	10	1	Pek _{vir}
Hetgly.G000027980	Pao retrotransposon peptidase family protein	–	21	18	3	88 _{vir}
Hetgly.G000028856	Serine/arginine repetitive matrix protein 1 isoform X1	–	5	5	0	Pek _{vir}
Hetgly.G000029665	Collagen alpha-3(VI) chain-like	–	12	9	3	88 _{vir}

^a Bold type indicates genes containing *condition specific* variants and genes with an (*) indicate putative effectors annotated to known plant-parasitic nematode effectors.

^b SignalP score predicts the presence of secretory signal peptides; (T) denotes genes containing a signal peptide with a transmembrane domain.

^c Virulence: individuals virulent on Peking (Pek_{vir}) and individuals virulent on PI 88788 (88_{vir}).

S-transferase (Hetgly.G000026104), a glutathione synthetase-like effector 22 (Gpa-GSS22–Hetgly.G000005243) and many genes annotated to putative gland expressed proteins. Based on these results, it is fair to hypothesize that alternative splicing might play a key role in virulence, by altering the coding sequence of effectors resulting in new gene functions or eliminating their recognition. These results are in line with those of previous works on the early life stage secretome of *H. glycines*. Focused on pre- and parasitic stage juvenile SCN, this study also identified isoforms in many predicted effectors including evidence of stage-specific preferential isoform usage (Gardner et al. 2018). Our findings would also suggest that the transcriptome is much more diverse and variable than predicted by the current reference genome (Masonbrink et al. 2019); with many putative isoforms, expressed under specific conditions, unreported. While our results give weight to the hypothesis of preferential isoform usage by Peking-virulent females, an in-depth investigation remains difficult. One such challenge resides in the redundancy of highly similar gene sequences throughout the transcriptome; vestiges of past duplication and recombination events (Masonbrink et al. 2019). Even with this single nematode-adapted RNAseq approach, limiting genetic diversity to one individual, short read sequencing may fall short at differentiating these highly similar genes and their isoforms. One possible alternative is the sequencing of mRNA using long-read technologies, which offers the advantages of capturing the entire gene sequence thus accurately discriminating between similar genes and their structural variants (Amarasinghe et al. 2020).

Investigating genes identified as containing SNPs and INDELs in all individuals of a phenotype, we noted a higher number of candidate genes in females developing on Peking than on PI88788. In Peking-virulent females, these included many unannotated putative effector genes as well as some annotated to known effectors such as an esophageal gland cell Hgg-20 protein (Hetgly.G000021578) and a dorsal gland cell-specific expression protein (Hetgly.G000027114). While less abundant in PI 88788-virulent females, a putative effector annotating to a pollen-specific leucine-rich repeat extensin-like protein (Hetgly.G000004483) was also identified alongside a predicted Mucin gene (Hetgly.G000021835). In both cases, most variants associated with these genes had only missense impacts on the resulting protein. This could suggest that effector evolution tends to modify these sequences, leading to neofunctionalization or detection avoidance, rather than rendering them nonfunctional.

Sequence variations in the biotin synthase gene *HgBioB* were identified by Bekal et al. (2015) and shown to produce an active protein in the avirulent SCN inbred strain TN10 and an inactive form in the virulent TN20 strain (Kwon et al. 2019). The authors screened field populations and found a mixture of both alleles in most of the samples, with the virulent allele being slightly more prevalent. They suggested that this could result from the recent increase in virulence in several fields but did not test the virulence profiles of these isolates. The most similar sequence found in our analysis and in the reference genome (Hetgly.T000006770.1) shared only ~70% homology with their sequences but did code for a biotin synthase. We found five mutations inside this gene but the genotypes varied greatly between individuals of the same phenotype and no correlation with virulence could be established. As mentioned by Kwon et al. (2019), the inactive form of the *HgBioB* could have been selected by the multiple rounds of inbreeding in TN20 and unrelated to virulence.

While our results show that gene regulation is affected in virulent nematodes and that sequence polymorphisms could play a role in the acquisition of nematode virulence against both Peking and PI 88788, alternative biological pathways leading to virulence should be further explored. One such possibility warranting further investigation is the manipulation of host root methylome via nematode secreted proteins. Indeed, looking across all analyses, our findings show the presence of two upregulated DEGs annotated

to histone methyltransferase genes (Hetgly.G000006629 and Hetgly.G000029269) in the Pek_{vir} versus Avir contrast. These are proteins known to be involved in DNA methylation; an epigenetic mechanism by which gene regulation is modulated through the recruitment of proteins involved in gene repression or by inhibiting binding of transcription factors to DNA molecules (Bartels et al. 2018). A recent study on the methylome of soybean roots has conclusively shown evidence of generalized DNA hypomethylation of syncytial cells in cases of compatible interactions with SCN (Rambani et al. 2015). With growing evidence that host methylome trafficking by phytoparasitic nematodes secreted proteins may alter host gene expression in favor of successful parasitism, this mechanism should be investigated in SCN compatible reaction with resistant soybean cultivars (Deleris et al. 2016; Hewezi 2020; Rambani et al. 2015, 2020). While still too early to conclude on the role of these genes, they offer tantalizing evidence of a previously unexplored pathway for the acquisition of virulence by SCN.

To our knowledge, this is the first use of a “single-cell” RNA sequencing approach to study gene expression in single individuals of plant-parasitic nematodes. Although the scope of this study only focused on the effectorome of J4 females, working with individual nematodes allowed for the identification of specific effector genes that are putatively associated with virulence on resistant soybean genotypes. Our analysis highlights the differences in virulent SCN individuals developing on both major soybean resistant sources.

ACKNOWLEDGMENTS

We thank Albert Tenuta (OMAFRA), Tom Welacky (AAFC), and Ontario soybean growers for providing SCN field populations, and Pierre-Yves Véronneau, Anatoly Belov, and Joël Lafond-Lapalme for technical and bioinformatic assistance.

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