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Gene expression analyses on *Dickeya solani* strains of diverse virulence levels unveil important pathogenicity factors for this species

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Dickeya solani **causes soft rot and blackleg mainly on potato crops. High pathogenicity of this species results from efficient production of plant cell wall-degrading enzymes, especially pectate lyases, potent root colonization, and fast vascular movement. Despite genomic homogeneity, variations in virulence-related phenotypes suggest differences in the gene expression patterns between diverse strains. Therefore, the methylomes and transcriptomes of two strains (virulent IFB0099 and low virulent IFB0223), differing in tissue maceration capacity and virulence factors production, have been studied. Methylation analysis revealed no significant differences. However, the analysis of transcriptomes, studied under both non-induced and induced by polygalacturonic acid conditions (in order to mimic diverse stages of plant infection process), unveiled higher expression of pectate lyases (*** ***pelD***** , ***** ***pelE***** , ***** ***pelL*****), pectin esterase (*** ***pemA*****), proteases (*** ***prtE***** , ***** ***prtD*****) and Vfm-associated quorum-sensing genes (*** ***vfmC***** , ***** ***vfmD***** , ***** ***vfmE*****) in IFB0099 strain compared to IFB0223. Additionally, the genes related to the secretion system II (T2SS) (*** ***gspJ***** , ***** ***nipE*****) displayed higher induction of expression in IFB0099. Furthermore, IFB0099 showed more elevated expression of genes involved in flagella formation, which coincides with enhanced motility and pathogenicity of this strain compared to IFB0223. To sum up, differential expression analysis of genes important for the virulence of *** ***D. solani***** indicated candidate genes, which might be crucial for the pathogenicity of this species.**

Keywords *Dickeya* spp., Blackleg, Methylomes, RNA-Seq, Soft rot, Transcriptoms

Abbreviations

CDS	Coding DNA sequence
DEGs	Differentially expressed genes
KDG	2-keto-3-deoxygluconate
MLST	Multilocus sequence typing
MTases	Methyltransferases
PCA	Principal component analysis
PCWDEs	Plant cell wall degrading enzymes
PFGE	Pulsed-field gel electrophoresis
PGA	Polygalacturonic acid
REP-PCR	Repetitive sequence-based PCR

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RIN	RNA integrity number
RSEM	RNA-Seq by expectation-maximization
SRP	Soft Rot *Pectobacteriaceae*
TSA	Tryptic Soy agar medium
VNTR	Variable number of tandem repeats

Bacteria classified within the genus *Dickeya* are responsible for economically significant plant diseases such as soft rot, which may be observed on many crops, vegetables or ornamentals, and blackleg of potatoes[1]. As a member of the Soft Rot *Pectobacteriaceae* (SRP), it belongs to a group of bacteria known for their ability to degrade plant cell wall components, leading to tissue maceration and characteristic symptoms on plants. Due to a notable impact on the yield and quality of agricultural products, *Dickeya* spp. were included in 2012 in the top ten list of most important bacterial plant pathogens[2]. In particular, *Dickeya solani* draw scientific attention as this species was a major causative agent of blackleg in Europe already in 2010[3]. Severe disease outbreaks were reported in Israel, with incidences ranging from 5 to 30% among five potato cultivars, affecting over 200 hectares of arable land[4]. Although majority of *D. solani* strains isolated from various environmental sources, including potato, ornamentals, and plant rhizosphere in Europe and Israel, demonstrate a high degree of genetic homogeneity[3,5,6], recent studies have shown that the genetic diversity of *D. solani* may be slightly larger than previously thought[7]. Specifically, a broader genomic diversity has been suggested by relying on characterization of three clades of strains isolated in Europe and Latin America. Basing on this data the diversity of *D. solani* may vary depending on the inhabited geographic region and/or environmental niche[7].

The competitive advantage of *D. solani* is associated with higher aggressiveness of these strains in contrast to the representatives of the other *Dickeya* spp. This pathogen requires lower optimal temperatures for disease development, and shows an ability to set up infection from relatively smaller initial inoculum[8,9]. Furthermore, *D. solani* strains seem to have a greater capacity to colonize the roots of potato plants and spread through the vascular system of the host[10]. Successful colonization of the plant demands involvement of additional elements facilitating bacterial movement or adhesion, i.e. flagella, lipopolysaccharides, and exopolysaccharides[11,12]. Most of all, the pathogenic potential of *Dickeya* spp. is linked with efficient production and secretion of numerous virulence factors, predominantly belonging to plant cell wall degrading enzymes (PCWDEs)[13]. Besides, a notable role is attributed to the intermediate factors allowing for bacterial adaptation to the conditions encountered within the host tissue, including oxidative stress, iron deficiency, and the presence of toxins[14].

In general, the symptoms of soft rot result from the action of PCWDEs, particularly pectate lyases (Pels), as these enzymes break down polygalacturonic acid (PGA), an important component of pectin, which builds up the plant cell wall, primary and middle lamella. In the *Dickeya* genus, genes responsible for catabolism of poli- and oligogalacturonides are regulated by the repressor KdgR, which becomes inactive during accumulation of 2-keto-3-deoxygluconate (KDG), an intermediate product of pectin degradation[13]. The expression of genes encoding major pectate lyases PelD and PelE is primarily induced by PGA in addition to the various associated side chains[15]. Although the presence of rhamnose stimulates the expression of *rhiE* gene, encoding rhamnogalacturonate lyase by RhaS-dependent regulation[16,17], the regulatory regions of *pelD* and *pelE* genes lack binding sites for RhaS[18]. In addition, the transcription of the gene of a secondary pectate lyase PelL, is induced by both the presence of PGA and plant extract[19]. Therefore, supplementation of the culture media with PGA mimics the advancement of the infection process and tends to be applied under laboratory conditions to induce the expression of genes encoding pectate lyases[13].

Additional PCWDEs, such as cellulases and proteases, enhance the ability of pectinases to break down the cell wall components. Most *Dickeya* spp. secrete one cellulase (Cel5Z) and up to four proteases (PrtG, PrtA, PrtB, PrtC). Furthermore, different protein secretion systems (T1SS to T6SS) are present in SRP. All *Dickeya* and *Pectobacterium* spp. strains are equipped with the type II secretion system (T2SS) Out system responsible for secretion of extracellular pectinases and cellulases[9]. These bacteria are also equipped with flagella, facilitating chemotaxis-driven movement towards the entry sites on plant surfaces[12].

Previously, numerous studies have examined the role of various regulatory genes in the pathogenicity of *Dickeya* spp. For instance, the *gacS*/*gacA* two component signal transduction system was pointed essential for the pathogenicity and systemic movement of *D. dadantii* 3937, as it significantly affected biofilm formation, exoenzyme production, and the expression of the genes linked to the type III secretion system (T3SS). The phenotype of a knockout mutant impaired bacterial ability to invade plants systemically and to cause disease[20]. On the other hand, PecS protein, first characterized in *Dickeya* spp. in 1994, has been identified as a major global regulator in *D. dadantii* 3937, as it controlled over 600 genes and played a crucial role in the early stages of infection by modulating the expression of genes enabling plant colonization and preventing premature induction of virulence genes[21–23]. The studies performed by Royet et al.[24] have shown that genes such as *opgG*, *opgH*, *rffG*, *kduI*, *acrA*, *acrB*, *guaB*, *carA*, *carB*, *mlaC*, *mlaF*, *tolC*, being the components of the TonB system, and chemotaxis-related genes (*cheA*, *cheR*, *cheW*, *cheY*, *cheZ*) are essential for the virulence and survival of *D. dadantii* 3937 in the plant environment. These genes play significant roles in processes ranging from osmoregulated synthesis of glucan and exopolysaccharide production to iron-siderophore transport and protein quality control, which underlines their importance in bacterial adaptation and pathogenicity within the host plants. In another study on a highly virulent *D. solani* Ds0432-1, it was found that mutation in a gene encoding a small regulatory RNA ArcZ switches off the antimicrobial activities of this bacterium against other microorganisms by these means limiting the competitiveness of this strain[25]. Brual et al.[25] pointed out that a polymorphism in *arcZ* is a key determinant for the observed variability in the virulence of diverse *D. solani* strains showing high genomic homogeneity.

In other studies on the *D. dadantii* 3937 strain, deletion of the *cpxA*/*cpxR* genes, which encode parts of a two-component regulatory system, revealed significant roles of these genes in controlling the expression of T3SS

components and biofilm formation, which tend to be pivotal for bacterial virulence[26,27]. This regulatory system is also involved in modulating stress responses, such as the one associated with envelope damage occurring during early stages of infection[26]. Moreover, the gene *hrpL*, regulated by the two-component system (TCS) HrpX/HrpY, is essential for expression of the T3SS genes, impacting the bacterial ability to infect plants. These findings have been confirmed in plant-involving assays, which highlights their importance for the virulence of *D. dadantii* 3937[27]. Last but not least, the Vfm quorum-sensing (QS) gene cluster, originally identified in *D. dadantii* 3937, also plays essential role in regulating virulence-linked gene expression *in planta*[28]. In *D. solani* IFB0223, the Vfm cluster is similarly essential for the QS mechanism, contributing significantly to the ability of the pathogen to infect and colonize potato plants, as demonstrated by the reduced pathogenicity of Vfm mutants observed *in planta*[29]. Finally, the Vfm QS system (VfmI, VfmH, VfmB, VfmE), MarR family regulators (PecS, OhrR, MfbR and SlyA), and Tsp systems (TspB, TspA) were revealed to be important for the virulence and competitive fitness of *D. dadantii* 3937, while the Vfm system regulated the expression of virulence genes *in planta* and the Tsp systems delivered toxins for the contact-dependent growth inhibition during plant infection[9,30].

Passing to *D. solani*, even the strains of diverse geographical origin and isolation year exhibit high genomic homogeneity despite significant differences in their virulence[31]. High uniformity in this taxon was evident in Repetitive sequence-based PCR (REP-PCR) or Pulsed-Field Gel Electrophoresis (PFGE) genomic profiling, besides low variation in the sequences of housekeeping genes as shown by Multilocus Sequence Typing (MLST) or whole-genome sequencing[3,6]. Similarly, a comparative genomic study including 22 *D. solani* strains revealed only minor differences in their genomic structures, contributions to pangenome fractions, ANIb, ANIm and Tetra values, in addition to the core genome-based phylogeny[6]. Moreover, Motyka-Pomagruk et al.[6] demonstrated that the genomes of the virulent strain IFB0099 and the low virulent strain IFB0223, deriving from potato-associated environments, exhibit high synteny, with an average nucleotide identity (ANI) of up to 100%. Despite this high level of similarity, specific differences have been identified between these strains, including a 3 kb deletion in IFB0223 comprising the region encoding a regulatory RNA ArcZ along with *mtgA*, *elbB*, and an unknown gene[25]. Additionally, previous studies reported ten single nucleotide polymorphisms (SNPs) between IFB0099 and IFB0223, with five leading to non-synonymous mutations[5]. Despite these genomic similarities, significant phenotypic differences between these strains were noted, particularly in terms of virulence factors production (pectinases, cellulases, and proteases), motility, and the ability to macerate plant tissue[5,31,32].

The differences in the virulence-related phenotypes among plant pathogenic bacteria can be related not only to the regulation of gene expression, but also may result from DNA methylation patterns that control the temporal expression of specific gene subgroups[33]. DNA undergoes methylation at specific positions on cytosine and adenine nucleotides, exactly at C-5 or N-4 sites of cytosine and the N-6 site on adenine. This process is carried out by enzymes called DNA methyltransferases (MTases) including cytosine MTase (e.g. Dcm of *Gammaproteobacteria*), adenine methylase (e.g. Dam in *Gammaproteobacteria*) and cell cycle-regulated methylase (e.g. CcrM in *Alphaproteobacteria*)[33]. In view of the methylome-associated research, Low et al.[34] demonstrated that the pathogenicity of Dam-deficient *Dickeya chrysanthemi* strain (there referred to as *Erwinia chrysanthemi*) was significantly diminished on two plant hosts i.e. African violet and lettuce.

Considering that our previous study on 22 *D. solani* genomes of strains isolated from various geographical locations and predominantly from potato hosts, did not reveal clear genetic determinants responsible for the observed variations in virulence among *D. solani* strains[6], we formulated the aims of the current study focused on application of methylation analysis and transcriptomic profiling to get an insight into the mechanisms of virulence of this economically significant phytopathogen. The presented research was performed on the virulent IFB0099 strain (isolated from rotten potato plant) and the low virulent IFB0223 strain (isolated from the healthy potato rhizosphere), which exhibited notable phenotypic differences, including variations in the production of virulence factors such as pectinases, cellulases, and proteases, as well as differences in motility and the ability to macerate plant tissue[5,31,32]. Therefore, the goal of the current study was to provide an insight into the methylomes of virulent (IFB0099) and low virulent (IFB0223) *D. solani* strains, supported by extensive transcriptomic profiling conducted under both induced (medium supplemented with PGA) and non-induced conditions (medium without PGA). The herein reported research pointed to several candidate genes that may contribute to explaining the differences in the pathogenic potential of *D. solani* strains.

Results

The DNA methylomes of *D. solani* IFB0099 and *D. solani* IFB0223

Methylation of genomic DNA is a well-established regulatory mechanism that can influence gene expression profile, also involving the genes coding for virulence factors, therefore ultimately contributing to the manifestation of disease symptoms. During our former study the genomes of *D. solani* IFB0099 and IFB0223 were sequenced on Pacific Biosciences platform[5], thus we here conducted an analysis on this data to identify differences in the DNA methylation patterns between the genomes of these two strains.

In total three methylated motifs were revealed in the genomes of *D. solani* IFB0099 and IFB0223, all of which were N6-methyladenosine (m6A) modifications (Table 1). Among these modifications, majority of the adenine methylations were observed at the 5'-GATC-3' site methylated by the Dam MTase, commonly identified in the members of the class *Gammaproteobacteria*. In addition, the following sites were methylated in both strains: 5'-CNCA(N7)RTGG-3' and the complement 5'-CCAY(N7)TGNG-3' in addition to 5'-GCA(N5)GTTC-3' and the complement 5'-GAAC(N5)TGC-3'. The frequencies of methylation of all three motifs were similar, albeit not identical, between the here investigated two strains.

Figure 1 shows the extent of methylation among all the identified methylation motifs within a 10 kb window in the two analyzed *D. solani* strains (IFB0099 and IFB0223). The genome-wide methylation patterns for strain IFB0099 (Fig. 1A-C) are comparable to those of the IFB0223 (Fig. 1D-F) strain. The 5'-GATC-3' motif shows robust methylation across the chromosome and population. Moreover, the level of 5'-GATC-3' methylation

Motif[a]	Type[b]	*Dickeya solani* IFB0099		*Dickeya solani* IFB0223	
		Count[c]	Fraction[d]	Count[c]	Fraction[d]
G**A**TC CT**A**G	m6A	22,571	0.97	22,592	0.95
CNC**A**(N7)RTGG GNGT(N7)Y**A**CC	m6A	1053 1053	0.98 0.97	1060 1060	0.93 0.94
GC**A**(N5)GTTC CGT(N5)C**A**AG	m6A	440 440	0.96 0.96	441 441	0.95 0.90

Table 1. Methylated motifs identified in the genomes of *D. solani* IFB0099 and IFB0223. [a]The methylated nucleotides are indicated in boldface font. [b]Marks whether the modification is N^6-methyladenoside (m6A). [c]The total number of appearances of the motif in the genome, regardless of the methylation status. [d]The relative frequency of a particular methylated motif in the analyzed genome.

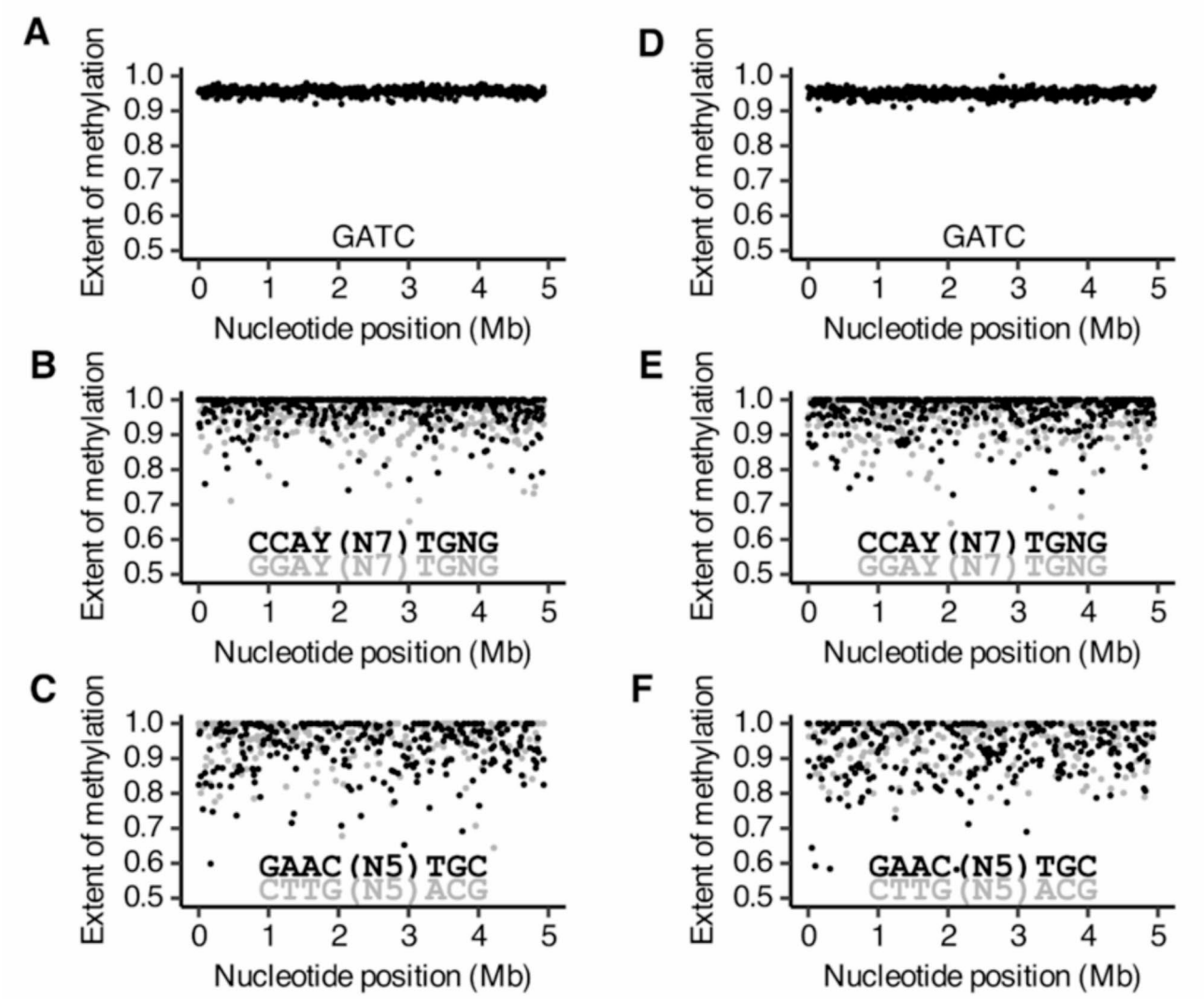

Fig. 1. Genome-wide DNA methylome of IFB0099 and IFB0223 *D. solani* strains. The extent of methylation is shown, using a 10-kb sliding window, of the methylated sites across the *D. solani* IFB0099 (**A** to **C**) and IFB0223 (**D** to **F**) chromosomes. Data for each of the three methylated motifs are shown in separate panels for every strain, with the sequence of each motif provided within the panels. In the case of non-palindromic motifs, in which each strand is methylated, the data for these two strands are shown in black (top strand) and grey (bottom strand).

ranged between 90 and 95%, suggesting that there is always a small fraction of bacterial cells that lack methylation at a given site (Fig. 1A and D). The other analyzed motifs show higher variation in the methylation rates. For most of the identified sites, the methylation is close to 100% (Fig. 1B, C, E and F).

No significant differences in the overall DNA methylation patterns were detected between the two *D. solani* strains, thus this analysis did not allow to explain the observed deviations in the virulence-associated phenotypes of IFB0099 in contrast to IFB0223. In order to further search for factors that might have an impact on the virulence of *D. solani*, the transcriptomes of IFB0099 and IFB0223 strains growing in non-induced (M9 without PGA in the medium) and induced (M9 with PGA in the medium) conditions were performed.

Transcriptomic profiling of *D. solani* IFB0099 and IFB0223

After quality trimming and adapter clipping, about 94% of the generated pairs of reads passed the quality checks and were subjected to further analysis (Table S1). On average, approximately 33 million reads were generated per library (Table S2).

Of the 31,883,758-35,397,968 paired-end reads per sample that passed the quality checks, more than 99% of the input was successfully mapped to the reference genome of *D. solani* IPO 2222, with over 66% of the reads aligning exactly once (Table S2). On the other hand, less than 0.59% of the paired-end reads did not align concordantly to the genome of *D. solani* IPO 2222 strain. Overall, these results indicated high quality of mapping of the reads, which ensures reliability of the subsequent analysis of differentially expressed genes (DEGs).

Overview of gene expression changes and principal component analysis of data generated for virulent (IFB0099) and low virulent (IFB0223) *D. solani* strains cultured under induced and non-induced conditions

Mean Average (MA) and Volcano plots were used to provide an overview of gene expression changes between the diverse culture conditions. No unusual patterns were detected in the MA or Volcano plots when comparing *D. solani* IFB0099 and IFB0223 in the induced vs. non-induced conditions (Figure S1).

To investigate the overall effects of the culture conditions (induced vs. non-induced) between two genotypes (IFB0099 or IFB0223) on the gene expression patterns in *D. solani*, Principal Component Analysis (PCA) was performed involving all the tested samples (Fig. 2).

The PCA plot shows the key sources of variation between the tested samples, with Principal Component 1 (culture conditions) explaining 61% of the variation in the dataset and Principal Component 2 (genotype) representing 14% of the variation. It is evident from the clustering in the PCA plot that the gene expression profiles are strongly influenced by the presence of PGA in the medium in which the strains were grown, while

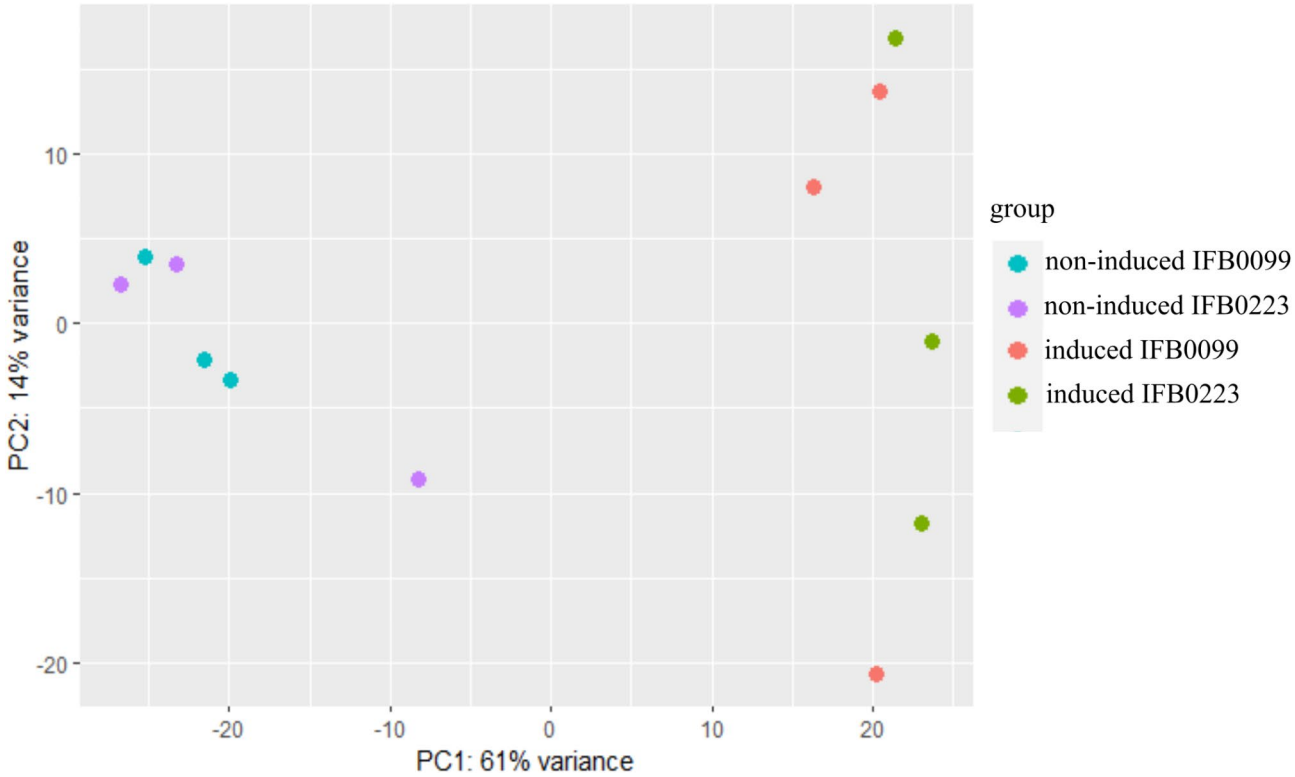

Fig. 2. Principal Component Analysis of the RNA-Seq data. The plot shows the two-dimensional similarity using the Bray-Curtis distance matrix. PC1 and PC2 represent the two principal components. Dots represent individual biological replicates and are colored according to the genotype and growth condition as shown within the in-figure legend.

the genotype had a much smaller contribution. Interestingly, there was noted a tighter clustering of the replicates from the non-induced conditions than the induced conditions along Principal Component 2, indicating that this source of variability predominated for the induced conditions (M9 + PGA).

Differentially expressed genes in *D. solani* IFB0099 and IFB0223

Differentially expressed genes in a statistically significant manner between D. solani IFB0099 and IFB0223 cultured in the induced or non-induced conditions

Initially, in order to identify genes that are differentially expressed in a statistically significant manner between *D. solani* strains IFB0099 and IFB0223, as analysed either under induced or non-induced conditions, calculations with DeSeq2 were performed. Regarding non-induced conditions, 81 upregulated genes and 75 downregulated genes were identified in *D. solani* IFB0099 compared to IFB0223 (Table S3). All the DEGs met the established thresholds for statistical significance (|Log2FoldChange| > 1 and adjusted *p*-value (*p* adj) < 0.05). Among these genes differing in expression in a statistically significant manner, only those of a potential functional impact on differences in the virulence of the analyzed *D. solani* strains were further studied and depicted in Table 2.

Gene name[a] GeneID[b]	Gene symbol[c]	Log2FoldChange[d]	*p* adj[e]
NON-INDUCED CONDITIONS (medium without PGA)			
Upregulated IFB0099 vs. IFB0223			
Serine endoprotease WP_013319029.1	*degP*	3.13	7.28E-04
Universal stress protein WP_024104057.1	*uspB*	1.92	1.34E-03
Protease inhibitor I42 family protein WP_013317564.1	-	1.44	0.02
Downregulated IFB0099 vs. IFB0223			
2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase WP_013318066.1	*kduD*	-1.51	0.01
Flagellar transcriptional regulator WP_013318527.1	*flhC*	-2.07	8.79E-03
Hcp family type VI secretion system effector WP_013318140.1	-	-2.09	0.02
Flagellar transcriptional regulator WP_013318528.1	*flhD*	-2.13	6.76E-03
Type VI secretion system PAAR protein WP_013317122.1	-	-2.29	0.04
Pectate lyase WP_237703426.1	*pelL*	-2.42	0.02
INDUCED CONDITIONS (medium with PGA)			
Upregulated IFB0099 vs. IFB0223			
Zinc, cobalt, and lead efflux system WP_081441327.1	*zntA*	2.53	2.87E-03
23S ribosomal RNA A4U42_RS08715	-	1.86	0.02
2-octaprenyl-6-methoxyphenol hydroxylase, FAD/NAD(P)-binding protein WP_013316186.1	*ubiH*	1.40	0.02
Downregulated IFB0099 vs. IFB0223			
Hypothetical protein WP_022632103.1	-	-1.51	3.8E-03
Glycosyltransferase family 4 protein WP_022632756.1	-	-2.30	4.87E-12
Tyrosine-protein kinase Wzc WP_013317070.1	*wzc*	-3.68	2.18E-12
23S ribosomal RNA A4U42_RS10175	-	-10.81	1.81E-158

Table 2. The selected genes differentially expressed in a statistically significant manner in *D. solani* IFB0099 in contrast to IFB0223. The selection criteria for these genes were |Log2FoldChange| > 1 and the adjusted *p*-value (*p* adj) < 0.05. [a]Gene name refers to the unique identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1). [b]Unique gene identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1). [c]Gene symbol based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1). [d]The Log2FoldChange value indicating the magnitude of deviation in expression of this gene between the *D. solani* IFB0099 compared to IFB0223. [e]The adjusted *p*-value, i.e. Benjamini-Hochberg corrected *p*-value, accounting for multiple testing and controlling the false discovery rate.

Table 2 aims to present DEGs of statistical significance between the virulent (IFB0099) and low virulent (IFB0223) strains of *D. solani* under both induced and non-induced conditions. These genes, although not directly linked to the major virulence factors, provide important insights into the broader metabolic and regulatory differences between these strains, contributing to a comprehensive understanding of the transcriptional landscape. Under the induced conditions, the conducted statistical analysis on gene expression levels identified three significantly upregulated genes (heavy metal translocating P-type ATPase, 23S ribosomal RNA, and 2-octaprenyl-6-methoxyphenol hydroxylase, FAD/NAD(P)-binding protein) and four significantly downregulated genes (hypothetical protein, glycosyltransferase family 4 protein, tyrosine-protein kinase Wzc, and 23S ribosomal RNA) in *D. solani* IFB0099 compared to IFB0223 (Table 2).

The here conducted analysis of the genes differentially expressed in a statistically significant manner between *D. solani* IFB0099 and IFB0223 under non-induced conditions, unveiled among the upregulated genes those encoding serine endoprotease DegP and universal stress protein UspB (Table 2). On the other hand, in the case of the downregulated genes in *D. solani* IFB0099 compared to IFB0223 under non-induced conditions, the lowered expression of the genes encoding proteins responsible for bacterial motility (FlhC and FlhD), metabolic processes involved in the breakdown and utilization of 2-dehydro-3-deoxy-D-gluconate (2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase KduD), pectin degradation (pectate lyase PelL) and building up the VI secretion system (Hcp family type VI), was shown (Table 2).

To further explore the biological processes associated with the observed differences in the gene expression patterns, Gene Ontology (GO) enrichment analysis was conducted for both strains under induced vs non-induced conditions by using DEGs filtered by an adjusted p adj < 0.05. For *D. solani* IFB0099 (Figure S2A), GO enrichment analysis highlighted the processes associated with biosynthetic and metabolic activities. Specifically, macromolecule, cellular, and aromatic compound biosynthetic processes were significantly enriched. The enrichment of these functional groups was accompanied by relatively high gene ratios, indicating that a substantial proportion of DEGs are involved in these biosynthetic pathways. In the case of *D. solani* IFB0223 (Figure S2B), the top enriched GO terms were related to metabolic adaptations, particularly those involving metabolism of organic acid and carboxylic acid in addition to lipid biosynthetic processes. These terms were also characterized by notable gene ratios, reflecting the involvement of a significant fraction of DEGs in the pathways related to metabolism of lipids and organic compounds.

Since the differences in the gene expression profiles between IFB0099 and IFB0223 strains were not huge and did not correlate with their distinct virulence profiles, GO enrichment analysis did not reveal any novel insights. Consequently, the analysis was refocused on comparing the impact of non-induced vs. induced conditions within each strain separately.

Comparison of the most significant DEGs under induced vs. non-induced conditions for D. solani IFB0099 or IFB0223 as analysed separately

Heatmaps of the normalized expression of 30 genes with the lowest adjusted *p*-value were computed to depict genes, regardless of the |Log2FoldChange| threshold, whose expression levels differed significantly in the virulent strain *D. solani* IFB0099 (Fig. 3) and low virulent strain IFB0223 (Fig. 4) under the induced (M9 + PGA) vs. non-induced (M9) conditions. The clear clustering of the three biological replicates from the induced and the non-induced experimental conditions (Figs. 3 and 4) confirms the reliability of the biological replicates of the transcriptomes of both *D. solani* IFB0099 and IFB0223.

In *D. solani* IFB0099, the gene showing the most statistically significant difference in expression between the induced vs. non-induced conditions encodes a protease inhibitor I42 family protein (Fig. 3). Among the other most statistically significant DEGs there were three transcriptional regulators: a gene coding for a LysR family transcriptional regulator, an AraC family transcriptional regulator (*yhiW*), and a gene encoding an AraC-type DNA-binding domain-containing protein. All three transcriptional regulators exhibited lowered expression in the induced compared to the non-induced conditions (Fig. 3). Additionally, a gene encoding a transcriptional regulator (*ahyR/asaR* family) was also identified among the most significant DEGs, showing a similarly reduced expression under the induced conditions. Interestingly, seven of the other most differently expressed genes encoded components of the transporter systems (two ABC transporter substrate-binding proteins, three transporter substrate-binding domain-containing proteins, putative urea ABC transporter substrate-binding protein and an amino acid ABC transporter permease). All of them showed a reduced expression in the induced conditions compared to the non-induced conditions (Fig. 3).

Among the 30 most varying in a statistically significant manner DEGs in IFB0223 between the analyzed conditions, there were several genes potentially associated with the virulence of SRP (Fig. 4). Like in IFB0099, a gene encoding a protease inhibitor I42 was among the top 30 most statistically significant DEGs in IFB0223, and in both strains, this gene showed the highest expression level under the induced conditions. Another example of a gene showing higher expression in the induced conditions is the one coding for a member of the TonB family, which includes outer membrane proteins involved in iron uptake in Gram-negative bacteria. Also, the *pehN* gene, coding for a polygalacturonase, exhibited higher expression in the induced compared to the non-induced conditions in *D. solani* IFB0223 (Fig. 4). As the induced conditions relied on addition of PGA, the noted higher expression of genes encoding polygalacturonases releasing digalacturonides from the polygalaturonic acid chains, may be related to availability of this specific substrate in the medium. Finally, *cpxP* gene, encoding a cell-envelope stress modulator involved in protection of bacteria from potentially toxic compounds, showed lowered expression under the induced conditions compared to the non-induced conditions.

Notably, several DEGs in *D. solani* IFB0099 under the induced compared to the non-induced conditions disclosed similar expression pattern as in the case of the IFB0223 strain. This observation concerns the gene encoding a protease inhibitor I42, which is overexpressed under induced compared to the non-induced conditions in both the analysed *D. solani* strains (Figs. 3 and 4). Also, the gene encoding the ABC transporter

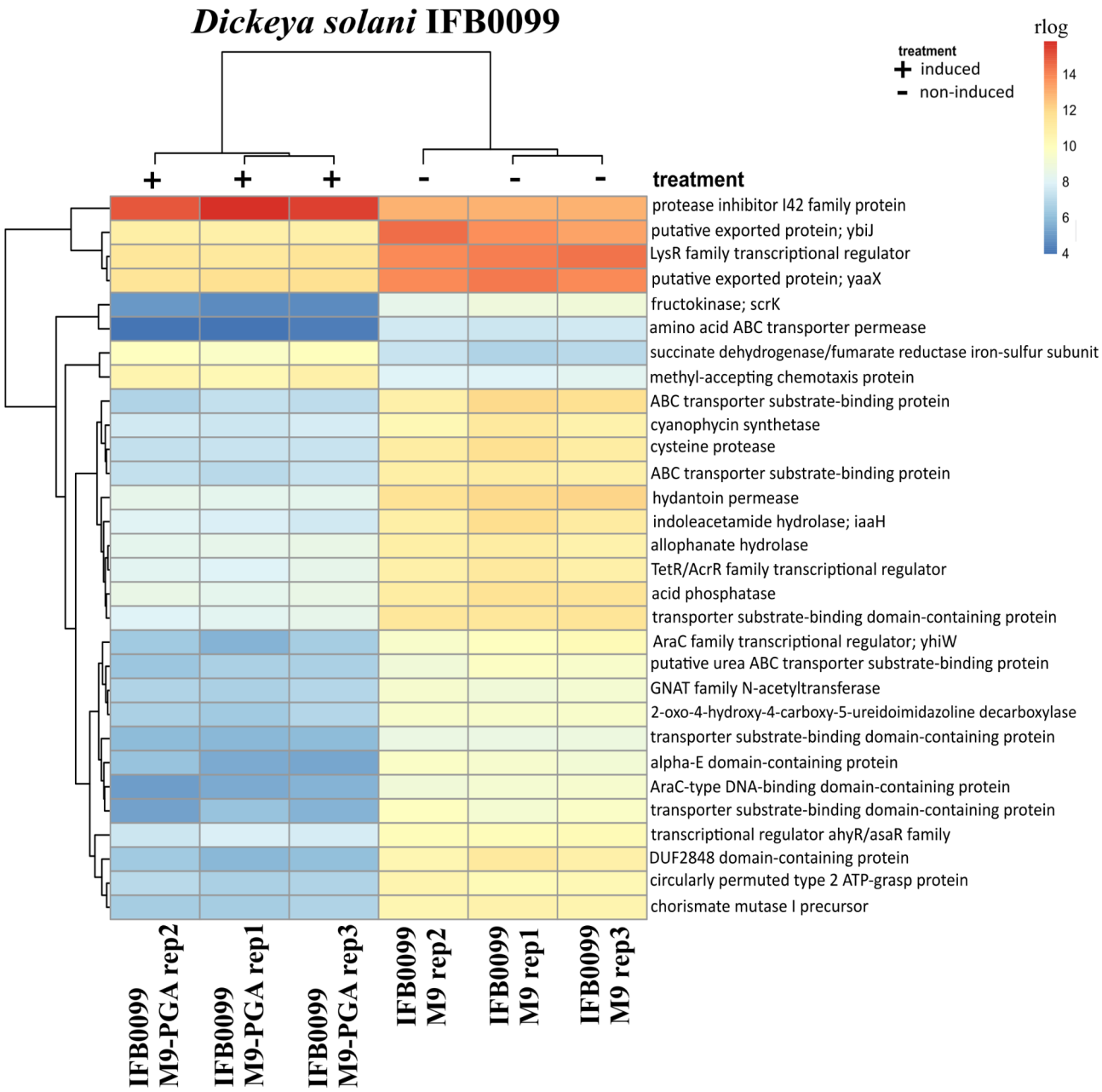

Fig. 3. Differential expression of 30 genes showing the lowest adjusted *p*-value for *D. solani* IFB0099. Hierarchical clustering was used to generate the heatmap. The scale represents the normalized expression level of the genes, which have been transformed using the regularized log technique (normalized log2-transformed expression values (rlog)). The selection criterion for these genes was the adjusted *p*-value (p adj) < 0.05.

substrate-binding protein exhibited differential expression between the studied conditions both in IFB0099 and IFB0223 (Figs. 3 and 4). Though, regarding IFB0099, a higher expression of this gene was observed in non-induced conditions, while in the case of IFB0223 in the induced conditions.

After providing an insight into the 30 most differentially expressed genes in a statistically significant manner between the induced vs. non-induced conditions in two strains of *D. solani*, we moved to examination of changes in the expression of genes, which are associated with the virulence of SRP.

Comparison of differentially expressed virulence-related genes between the induced and the non-induced conditions in D. solani IFB0223 and IFB0099

In *D. solani* IFB0099, 434 genes showing highly induced expression and 407 genes showing lowered expression were identified in the induced vs. the non-induced conditions with the established threshold for statistical significance of differential expression (absolute Log2FoldChange > |1|, p adj < 0.05) (Table S4). Concerning *D. solani* IFB0223, 380 and 363 genes were observed to be notably upregulated and downregulated, respectively,

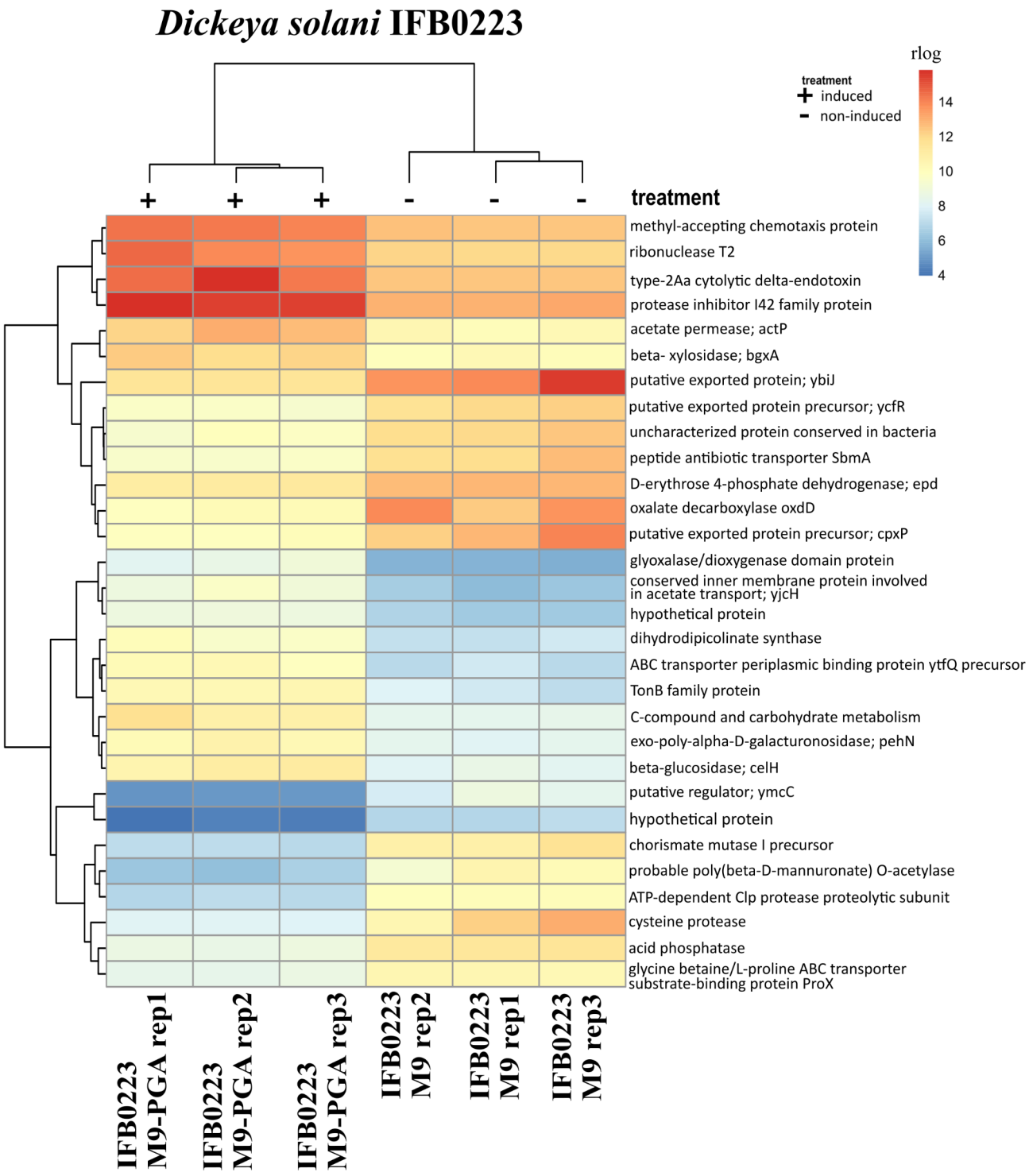

Fig. 4. Differential expression of 30 genes showing the lowest adjusted *p*-value for *D. solani* IFB0223. Hierarchical clustering was used to generate the heatmap. The scale represents the normalized expression level of the genes, which have been transformed using the regularized log technique (normalized log2-transformed expression values (rlog)). The selection criterion for these genes was the adjusted *p*-value (p adj) < 0.05.

if the strain had been cultured under induced in contrast to the non-induced conditions assuming the above-mentioned threshold for statistical significance (Table S5). In *D. solani* IFB0099, a total of 841 genes showed significant differential expression in response to PGA (434 genes upregulated and 407 genes downregulated), representing approximately one-fifth of the genome. Similarly in *D. solani* IFB0223, 743 genes were differentially expressed (380 upregulated and 363 downregulated) under the same conditions, also constituting a substantial portion of the genomic annotations.

Focusing our analysis on the genes known to be related to the virulence of SRP (Table 3), we decided to group the DEGs between induced and non-induced conditions in *D. solani* IFB0099 and/or IFB0223 into the following categories: genes encoding pectinases involved in polygalacturonic acid and pectin degradation, genes coding for proteins involved in degradation of KDG, genes related to the synthesis and secretion of proteases, genes associated with T2SS, and the genes encoding flagellar proteins. For the analysis of differential expression of genes encoding major virulence factors in the case of IFB0099 (induced vs. non-induced conditions) and IFB0223 (induced vs. non-induced conditions), all genes were included, regardless of their statistical significance.

In terms of both IFB0099 and IFB0223 *D. solani* strains, the presence of PGA induced expression of genes encoding endo-pectate lyases (*pelD*, *pelE*, *pelI*, *pelB*, *pelL*, *pelC*), a pectin acetyl esterase (*paeY*) and pectin methyl esterases (*pemA*, *pemB*), which are involved in degradation of pectin, an important component of the plant cell wall. Of the above-mentioned genes, *pelD*, *pelE*, and *pelL* showed higher levels of induction in the presence of PGA in the virulent strain (IFB0099) compared to the low virulent one (IFB0223). Similarly, *paeY* (encoding a pectin acetyl esterase) showed greater induction in IFB0099 than in IFB0223. The two genes encoding pectin methyl esterase displayed contrasting patters, with *pemA* showing stronger induction in IFB0099 compared to IFB0223, whereas the opposite observation was true for *pemB* (Table 3).

Interestingly, the gene of exo-polygalacturonase (*pehV*) showed reduced expression in both *D. solani* strains if they were cultured in the presence of PGA (Table 3). Curiously, the repression of this gene was much weaker in IFB0099 (the virulent strain) compared to IFB0223; the log2FC for IFB0223 equaled -1.24, while for IFB0099 it was -2.85. It is worth pointing out that the endo-polygalacturonase encoded by *pehN*, which was identified among 30 most statistically significant DEGs in IFB0223 (Fig. 4), was also similarly upregulated in the other strain cultured with addition of PGA (Table 3).

The group of PCWDEs that contributes to the virulence of SRP includes proteases. Among main three proteases (PrtA, PrtD, PrtE) produced by *Dickeya* spp., the *prtA* gene showed strong and equal upregulation in both *D. solani* strains. On the other hand, *prtD* and *prtE* genes turned out to be notably induced by PGA only in the virulent strain IFB0099. Concerning *degQ* and *degP*, encoding serine endoproteases belonging to the group of Htr proteases, these genes showed a reduced expression in the induced compared to the non-induced conditions in both *D. solani* strains, with *degP* showing a much larger decrease in IFB0223 compared to IFB0099 (Table 3).

Another genes *kdgM* and *kdgN*, both coding for transmembrane porins involved in transport of oligogalacturonates, were highly upregulated in the presence of PGA in both IFB0099 and IFB0223. Interestingly, *kdgM* showed greater induction of expression in the virulent (Log2FoldChange 5.60) than the low virulent strain (Log2FoldChange 3.77), whereas the opposite was true for *kdgN*. In terms of the genes encoding proteins involved in the breakdown of galacturonate, *kdgA* and *kdgF* displayed somewhat greater induction in IFB0099 than IFB0223 (Table 3).

Next, we examined the expression of genes related to the T2SS involved in the secretion of pectinases and cellulases. In terms of T2SS effectors, a gene encoding necrosis-inducing protein NipE, exhibited higher expression under induced compared to the non-induced conditions in both IFB0099 and IFB0223. Notably, *nipE* showed higher induction in the virulent strain (IFB0099) compared to the low virulent strain (IFB0223). On the other hand, the expression of the T2SS building block *gspJ* was reduced in the presence of PGA in both *D. solani* strains, though the reduction was stronger in IFB0223 compared to IFB0099 (Log2FoldChange for IFB0099 amounted -0.54 and for IFB0223 equaled -1.26). Similarly, the expression of the *gspI* and *gspK* genes, encoding other components of T2SS, was also decreased under induced conditions in both strains, but to a greater extent in the virulent strain IFB0099 (Table 3).

Other differences identified between the transcriptomes of IFB0099 and IFB0223 cultured under induced vs. non-induced conditions, turned out to be associated with bacterial movement. From this functional group there were revealed ten genes encoding flagellar proteins, i.e. *flgC*, *flgB*, *fliE*, *flgD*, *flgG*, *flgF*, *flgE*, *fliF*, *flhD*, and *flhC* that showed higher expression in both strains if they were grown in the induced compared to the non-induced conditions. Passing to the extent of upregulation of the above-listed genes, only minor differences were observed between IFB0099 and IFB0223 in this matter (Table 3).

Differential expression of regulatory genes controlling virulence and metabolism in D. solani IFB0099 and IFB0223 cultured under induced vs. non-induced conditions

While studying differences in the expression of regulatory genes between *D. solani* strains IFB0099 and IFB0223, we examined the expression patterns of several key regulators involved in the control of virulence and metabolism in SRP. The investigated genes included regulators specific to pectin metabolism (e.g. *kdgR*, *kdgK*, *kdgF*, *kdgA*), two-component systems (GacS/GacA, CpxA/CpxR, HrpX/HrpY), QS systems (Vfm, ExpI/ExpR), post-transcriptional regulators (RsmA/RsmB), global regulators of metabolism and virulence (CRP-cAMP), and stress-related regulators (e.g. ohrR, slyA). Additionally, the expression of genes associated with DNA methylation (Dam methylase) and coding for small regulatory RNAs (e.g. ArcZ) were included in this research. The detailed outcomes of DEGs for all the above-mentioned genes are presented in Table S6.

Among the regulatory systems analyzed, the most notable results were linked to the Vfm QS system, which regulates the production of virulence factors such as PCWDEs, bacterial motility, and their ability to macerate plant tissues. This comparison was made between the induced and the non-induced conditions, highlighting the differential regulatory effects under these contrasting culturing approaches. The genes *vfmC*, *vfmD*, *vfmB*, and *vfmE* exhibited significantly higher expression under the induced conditions compared to the non-induced conditions in both strains, with the expression levels being notably higher in IFB0099 than in IFB0223. The most pronounced differences were observed for *vfmE*, with a Log2FoldChange of 3.87 in IFB0099 and 4.80 in IFB0223. These findings indicate higher activity of the Vfm system in strain IFB0099. Other regulatory systems, such as ExpI/ExpR and RsmA/RsmB, showed no significant differences in the level of expression when comparing the

Gene name[a] GeneID[b]	Gene symbol[c]	Log2FoldChange[d]		*p* adj[e]		Statistical significance[f]	
		IFB 0099	IFB 0223	IFB 0099	IFB 0223	IFB 0099	IFB 0223
Genes encoding pectinases							
Pectate lyase WP_013318983.1	*pelD*	4.66	4.20	NA	1.20E-04	NA	S
Pectate lyase WP_013318982.1	*pelE*	3.44	2.99	NA	2.24E-07	NA	S
Exo-poly-alpha-D-galacturonosidase WP_033111690.1	*pehN*	2.51	2.94	1.11E-16	4.73E-17	S	S
Endo-pectate lyase WP_013318842.1	*pelI*	2.46	3.20	NA	0.01	NA	S
Pectin esterase WP_013318985.1	*pemA*	1.92	1.79	9.51E-04	3.24E-05	S	S
Pectate lyase WP_013319745.1	*pelB*	1.68	3.30	1.40E-03	4.61E-06	S	S
Pectate lyase WP_237703426.1	*pelL*	1.66	1.21	2.99E-07	0.19	S	NS
Pectin acetylesterase WP_013318984.1	*paeY*	1.57	0.84	0.03	0.09	S	NS
Possible pectinesterase WP_013317553.1	*pemB*	1.46	1.94	0.01	4.62E-04	S	S
Pectate lyase WP_013319746.1	*pelC*	1.08	2.42	0.03	1.40E-03	S	S
Exo-poly-alpha-D-galacturonosidase precursor WP_013319862.1	*pehV*	-2.85	-1.24	2.83E-07	0.16	S	NS
Genes encoding proteins involved in degradation and secretion of 2-keto-3-deoxygluconate (KDG)							
Oligogalacturonate specific porin WP_013318071.1	*kdgM*	5.60	3.77	7.47E-04	4.0E-04	S	S
Oligogalacturonate specific porin WP_013317881.1	*kdgN*	2.66	3.77	2.87E-02	3.96E-04	S	S
Pectin degradation protein WP_013318064.1	*kdgF*	2.36	2.01	3.20E-03	3.41E-4	S	S
2-dehydro-3-deoxyphosphogluconate aldolase WP_013317813.1	*kdgA*	1.21	0.28	0.02	0.67	S	NS
2-dehydro-3-deoxygluconate kinase WP_013315827.1	*kdgK*	-1.73	-1.33	NA	0.09	NA	NS
Genes encoding proteases and involved in their secretion							
Secreted protease A precursor WP_013317949.1	*prtA*	2.35	2.43	1.09E-07	0.01	S	S
Proteases secretion protein WP_013317953.1	*prtE*	1.35	0.06	1.43E-05	0.94	S	NS
Alkaline protease secretion ATP-binding protein WP_013317954.1	*prtD*	1.17	0.47	3.29E-04	0.52	S	NS
Serine endoprotease WP_013316021.1	*degQ*	-1.93	-2.06	1.61E-06	7.86E-05	S	S
Serine endoprotease membrane-associated WP_013319029.1	*degP*	-2.3	-4.81	8.81E-09	2.29E-07	S	S
Genes encoding proteins associated with type II secretion system (T2SS)							
Necrosis-inducing protein WP_013318773.1	*nipE*	1.49	0.48	0.03	0.74	S	NS
Type II secretion system minor pseudopilin WP_013318805.1	*gspI*	-0.20	-1.15	0.67	0.02	NS	S
Type II secretion system minor pseudopilin WP_013318341.1	*gspJ*	-0.54	-1.26	0.13	0.03	NS	S
Type II secretion system minor pseudopilin WP_013318340.1	*gspK*	-0.55	-1.10	0.27	0.11	NS	NS
Genes encoding flagellar proteins							
Flagellar basal-body rod protein WP_012769280.1	*flgC*	2.02	1.69	8.73E-06	NA	S	NA
Flagellar basal-body rod protein WP_013318510.1	*flgB*	2.01	1.83	4.09E-06	0.07	S	NS
Flagellar hook-basal body complex protein WP_013318487.1	*fliE*	1.84	1.81	4.62E-06	0.05	S	S
Flagellar basal-body rod modification protein WP_013318509.1	*flgD*	1.83	1.55	9.03E-04	NA	S	NA
Flagellar basal-body rod protein WP_013318506.1	*flgG*	1.78	1.53	1.1E-03	0.18	NS	NS

Continued

Gene name[a] GeneID[b]	Gene symbol[c]	Log2FoldChange[d]		*p* adj[e]		Statistical significance[f]	
		IFB 0099	IFB 0223	IFB 0099	IFB 0223	IFB 0099	IFB 0223
Flagellar basal-body rod protein WP_013318507.1	*flgF*	1.75	1.43	6.5E-03	0.26	NS	NS
Flagellar hook protein WP_013318508.1	*flgE*	1.66	1.72	5.6E-03	NA	S	NA
Flagellar M-ring protein WP_013318488.1	*fliF*	1.38	1.54	4.49E-04	0.07	S	NS
Flagellar transcriptional activator WP_013318528.1	*flhD*	1.18	1.74	6.12E-05	0.02	S	S
Flagellar transcriptional activator WP_013318527.1	*flhC*	0.99	1.63	3.43E-03	0.04	S	S

Table 3. The selected differentially expressed genes (DEGs) possibly involved in the pathogenicity of virulent (IFB0099) and low virulent (IFB0223) *D. solani* strains under induced vs. non-induced conditions. The selection criterion: DEGs. [a]Gene name refers to the unique identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1). [b]Unique gene identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1). [c]Gene symbol based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1). [d]The Log2FoldChange value indicating the magnitude of deviation in the expression of this gene either in *Dickeya solani* IFB0099 or IFB0223 between the induced compared to the non-induced conditions. [e]The adjusted *p*-value, i.e. Benjamini-Hochberg corrected *p*-value, accounting for multiple testing and controlling the false discovery rate. [f]Statistical significance determined on the basis of *p* adj (p adj < 0.05). 'S' refers to a statistically significant difference and 'NS' to lack of statistically significant differences in gene expression between the induced compared to the non-induced conditions. Additionally 'NA' means not available.

induced vs. the non-induced conditions for growing of each strain. The CpxA/CpxR system and the global regulators CRP-cAMP and HexA/PecT displayed varying expression patterns for the individual genes, but no consistent trends which might result in varying virulence of these strains were observed.

In summary, the examination of changes in the expression of individual genes with special focus on the ones associated with the virulence of SRP revealed important discrepancies in the gene expression patterns in virulent IFB0099 vs. low virulent IFB0223 strain.

Discussion

The results of this study demonstrate that despite the high genomic homogeneity within the *D.solani* species, as previously reported with only 8.1% of genes belonging to the unique pangenome fraction[6], significant differences in gene expression profiles can explain the observed variations in the virulence between the virulent IFB0099 and low virulent IFB0223 strains. Notably, the IFB0223 strain, which was previously characterized as low virulent and isolated from the rhizosphere of a healthy potato[31], exhibited similar to the virulent IFB0099 strain, distinct transcriptional responses under the induced (PGA-supplemented) and the non-induced conditions.

The conducted genome-wide methylation analysis on virulent (IFB0099) and low virulent (IFB0223) *D. solani* strains revealed three m6A-type methylation motifs (5'-GATC-3', 5'-CNCA(N7)RTGG-3', 5'-GAAC(N5)TGC-3') in both strains. This data is consistent with the reports on the highest frequency of this type of methylation in the bacterial kingdom[35]. Interestingly, methylation of 5'-GATC-3' is a common motif among the representatives of the Gammaproteobacteria class, comprising *D. solani*, owing to Dam MTases[36]. Even though no significant variations in the DNA methylation patterns between *D. solani* IFB0099 and IFB0223 were noted, the robust methylation of 5'-GATC-3' across both chromosome and population agrees with the former data collected for other bacterial species[37]. A linkage between adenine methylation and bacterial virulence was suggested before for the plant pathogenic bacterium *Ralstonia solancearum*[38]. In that research, both m6A and m4C methylations occurred more often in a low virulent *R. solanacearum* strain compared to a highly virulent one. Moreover, the work of Bourgeois et al.[37] indicated that majority of DNA methylation in *Salmonella enterica* serovar Typhimurium remained static, even if the bacteria were cultured under significantly varying conditions that led to extensive changes in their transcriptomes. Furthermore, even if the alterations occurred in the methylation of individual bases, these changes generally did not correspond to variations in the gene expression profiles[37].

Focus on the differential expression of genes in the two *D. solani* strains cultured either under non-induced or induced conditions at the point of a stationary phase of growth unveiled changes in the gene expression patterns not only between different growth conditions, which was expected according to the earlier studies[13], but also among the two examined *D. solani* strains, IFB0099 and IFB0223 of a varying virulence potential.

The conducted transcriptomic analysis revealed a substantial number of DEGs in both strains when comparing induced and non-induced conditions. In the virulent strain IFB0099, 434 genes were upregulated and 407 were downregulated, while in the low virulent strain IFB0223, 380 genes were upregulated and 363 were downregulated under induced conditions (M9 with PGA in the medium) compared to non-induced conditions (M9 without PGA in the medium). These numbers correspond to approximately one-quarter of the genomic annotations in each strain. These findings highlight a substantial response to PGA on the level of the

transcriptome in both *D. solani* strains. Comparatively, in *D. dadantii* 3937, Jiang et al.[11] reported that 34% of the annotated coding DNA sequences (CDS) were differentially expressed under similar conditions (M63 + PGA medium), indicating a resembling, yet slightly more extensive gene response in a closely-related species from the family *Pectobacteriaceae*.

Besides investigating the influence of the medium composition on the gene expression pattern in *D. dadantii* 3937, Jiang et al.[11] performed the analyses both during exponential and stationary phases of bacterial growth. In the case of PGA supplementation and the stationary phase, the numbers of the induced and repressed genes were higher compared to our study (682 and 846, respectively; |FoldChange| > 2, FDR < 0.001). The production of virulence factors, in particular pectinolytic enzymes in SRP, depends notably on the bacterial growth phase and underwent investigation in multiple pieces of research. Importantly, majority of studies demonstrated that the activities of pectinases, proteases and a cellulase in *Erwinia carotovora* reached maximum in the stationary phase[9]. Potrykus et al.[39] revealed that the activity of pectate lyases in the potato tissue macerated by *D. solani* had a peak at 48 h post-infection, followed by a decline at 72 h. However, Jafra et al.[40] observed significant differences in the activity of pectate lyases in *D. dadantii* 3937 (formerly *Erwinia chrysanthemi* 3937) after 20 h from inoculation of potato tubers, with *pelE* and *pelI* exhibiting the highest expression levels, while the expression of *pelA* was moderate. It is worth to consider that in *D. dadantii* 3937, the expression of *pelA*, *pelD* and *pelE* genes changed differently in response to environmental stimuli relevant to plant infection, such as oxidative or osmotic stress[9]. The PCA analysis performed in the present study demonstrated that the culture conditions had a more significant impact on gene expression than the genetic differences between the examined strains.

Moving to the transcriptomes of *D. solani* IFB0099 and IFB0223, it is worth highlighting that stimulation of expression of three genes of endo-pectate lyases (*pelD*, *pelE*, *pelL*) and a gene coding for a pectin acetyl esterase (*paeY*) was significantly higher in the presence of PGA in the virulent strain IFB0099 compared to the low virulent strain IFB0223. Additionally, the genes encoding proteins involved in the secretion of proteases (*prtE* and *prtD*) exhibited elevated expression in the virulent strain. This observation is in accordance with the earlier data showing very low proteases activity in the low virulent *D. solani* IFB0223[5,31]. The current outcomes of transcriptomic profiling are in line with the research on *D. dadantii* 3937 and *D. solani* 3337 mutants affected in proteases production that at the same time exhibited the reduced virulence on saintpaulia or potato, respectively[9].

Besides, we pointed out that DEGs involved in the catabolism of oligogalacturonides, including the transmembrane porin *kdgM*, engaged in transferring extracellular unsaturated oligogalacturonides generated from pectin degradation into the periplasmatic space[41], were highly upregulated in the presence of PGA in the IFB0099 strain compared to IFB0223. Also, the other genes engaged in the decomposition of unsaturated galacturonates, i.e. *kdgA* and *kdgF*, turned out to be expressed more efficiently in IFB0099 than in IFB0223 post-induction with PGA. The putative roles of the constituents of the KDG pathway in the virulence of *Dickeya* spp., were presented earlier in the studies on *kdg* mutants of a closely related *D. dadantii* 3937, which turned out to be less competitive during infection of chicory leaves than the wild type strain[24]. Interestingly, the *kdgN* gene, whose expression was stimulated by PGA in both *D. solani* strains, showed higher induction in IFB0223 rather than IFB0099. This gene was revealed to be downregulated during early stages of infection (8 hpi) when the pathogen penetrates into the plant tissue as shown in the *D. dadantii* model[42].

The pectinases of *Dickeya* spp. are secreted *via* T2SS[9]. Here, a gene coding for a necrosis-inducing protein (*nipE*), a member of the T2SS, showed higher expression under induced conditions compared to the non-induced conditions in both IFB0099 and IFB0223, with greater induction in IFB0099 than in IFB0223. This data agrees with the findings of Mattinen et al.[43] that disclosed lower efficiency of *Pectobacterium parmentieri* SCC3193 (formely *Pectobacterium carotovorum* 3193) *nipE* mutant in macerating potato tuber tissue. Other gene *gspJ*, encoding a T2SS pseudopilin, showed lower expression in low virulent strain IFB0223 under induced conditions compared to the non-induced conditions. Furthermore, the genes coding for GspI and GspK, forming a complex with GspJ that enables the transport of proteins across the bacterial outer membrane, exhibited lesser reduction in expression in the virulent strain compared to the low virulent one also in other studies[44]. These diverse expression rates of the genes coding for the components of T2SS might be associated with different virulence of *D. solani* strains, as before *gspJ* was shown to be required for the pathogenicity of *Burkholderia cenocepacia*[45].

Other interesting outcomes include DEGs associated with bacterial movement. We noted higher expression of ten genes encoding flagellar proteins in both *D. solani* strains under induced in contrast to the non-induced conditions. Notably, the expression of six (*flgC*, *flgB*, *fliE*, *flgD*, *flgG*, *flgF*) of these genes showed higher stimulation in IFB0099 compared to the low virulent IFB0223. The presented results are in accordance with those demonstrating high expression of flagella-related genes during tuber maceration by another SRP species *Pectobacterium brasiliense*[46]. An important support pointing to the role of motility in bacterial virulence was provided by Antunez-Lamas[12], who showed that mutations in *motA* and *cheY*, involved in movement and chemotaxis, respectively, impaired the virulence of *D. dadantii* 3937 towards several plant hosts. Besides, Golanowska et al.[5] reported that the low virulent *D. solani* IFB0223 is less motile than IFB0099. The here presented data supports the thesis on the contribution of flagellar proteins to the differences in the virulence between the studied *D. solani* strains of various pathogenic potential. However, at the same time, we observed higher overexpression of *flhC* and *flhD* genes, coding for the components of the FlhD/FlhC complex, a transcription activator for the flagellar operons[47], in low virulent IFB0223 that in IFB0099 under the induced conditions compared to the non-induced conditions.

The Vfm QS system plays a pivotal role in regulating virulence factors production in bacteria from the genus *Dickeya*, including *D. solani*[28,29]. In our study, the genes from the Vfm system, particularly *vfmC*, *vfmD*, *vfmB*, and *vfmE* showed significantly higher expression level in IFB0099 strain compared to IFB0223 under both PGA-induced and non-induced conditions. Notably, *vfmE* a key component of this system, exhibited the most pronounced difference in expression, indicating greater Vfm activity in the virulent strain (IFB0099). These findings are consistent with the previous studies demonstrating that mutations in the *vfm* genes significantly

reduced the production of PCWDEs and the ability of bacteria to macerate plant tissues[29]. The stationary growth phase conditions (48 h) applied in our experiments further emphasized the importance of the Vfm system, as the QS regulates at this stage the responses of bacterial community assuring their survival and aggravating the severity of disease symptoms[28]. Additionally, genetic polymorphisms within *vfmO* and *vfmP* may have influenced the specificity of QS signals, as suggested by the earlier research[48]. Last but not least, the Vfm gene cluster is essential for QS in *D. solani*, significantly contributing to virulence and the ability of this pathogen to infect and colonize potato plants, as demonstrated by the reduced pathogenicity of Vfm mutants *in planta*[27,29]. The higher activity of the Vfm system revealed for IFB0099 under the induced conditions may thus be one of the reasons explaining the greater virulence of this strain compared to IFB0223.

In the context of *D. solani* environmental distribution, this species is considered to have a relatively narrow host range and majority of the so-far described strains originate from rotten potato plants or tubers with only a limited number of isolates acquired from the ornamentals (hyacinth and muscari), tomato or the rhizosphere of healthy plants[7]. The herein analysed IFB0099 strain has been isolated from a diseased potato stem in Poland, while IFB0223 has been obtained from the rhizosphere of a healthy potato plant in Germany[3,5,6,31,49]. In the rhizosphere these pectinolytic bacteria frequently function as saprotrophs and their pathogenic potential remains dormant until being triggered by factors such as high humidity or low oxygen availability[50]. Contrarily, when bacteria enter into the plant tissue and reach the necessary population density induction of the expression of key virulence genes (e.g. pectate lyases, cellulases) and their secretory pathways results in rapid maceration of the plant tissue and further spread of the infecting agents[10]. Differences in the expression of genes encoding the above-mentioned enzymes and regulatory systems (e.g. Vfm) may therefore influence the bacterial colonization strategy, competitiveness and ability to cause disease symptoms. As a result, virulent strains, such as IFB0099, may macerate host tissues in a more efficient manner[3,5,31]. Additionally, the DEGs between the virulent (IFB0099) and low virulent (IFB0223) strains of *D. solani* revealed under both induced and non-induced conditions, i.e. these involved in stress response (*uspB*), motility (*flhC*, *flhD*), and metabolic processes (*kduD*), may contribute to the environmental adaptability and fitness of *D. solani* strains, putatively explaining their diverse pathogenic potentials[14].

In conclusion, the here presented study provides a comprehensive transcriptome profiling of two *D. solani* strains IFB0099 and IFB0223, which differ in their virulence levels. Despite the lack of deviations in the DNA methylation, there were noted variations in the PGA-driven stimulation of expression of several of the virulence-associated genes between the analyzed strains. In more detail, the expression profiles of the genes encoding pectate lyases, proteases, and the components of flagella, as well as those linked with T2SS, are upregulated in the virulent *D. solani* IFB0099 to a higher extent than in the low virulent IFB0223 under the tested conditions. Furthermore, the genes coding for the components of the Vfm QS system, including *vfmE*, exhibited significantly higher expression under the induced conditions in IFB0099 compared to IFB0223. This observation underlines the essential role of the Vfm system in modulating virulence-associated traits and its potential as a target for the further functional research. Finally, as a result of the current study, several candidate genes have been pointed out that should be further investigated by the means of constructing deletion mutants and their subsequent compensation to obtain clear answers on the roles of the herein pointed DEGs in the virulence of an economically significant, necrotrophic phytopathogen *D. solani*.

Materials and methods

Bacterial cultures

Two strains of *D. solani*, differing notably in virulence were selected for the current research. *D. solani* IFB0099 was isolated in Poland in 2005[51] from a rotten potato plant. The strain is characterized by high aggressiveness and pathogenicity on potato plants, leading to significant tissue maceration and symptoms of blackleg and soft rot. On the other hand, *D. solani* IFB0223 was isolated in 2005 from the rhizosphere of a healthy potato plant in Germany, and it exhibits low maceration potency on both potato tubers and chicory leaves[5,31]. This strain exhibits a reduced virulence compared to IFB0099 and is considered less aggressive, making it a useful comparative model for pathogenicity studies. The genomic sequences of *D. solani* IFB0099 and IFB0223 have been deposited in the GenBank database under the accession numbers CPO24711 and CPO24710, respectively[5]. *D. solani* IFB0099 and IFB0223 are available in the bacterial collection of Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk (IFB UG & MUG) in Poland, in which they are stored at -80 °C in 40% (v/v) glycerol.

The biomass of *D. solani* strain (either IFB0099 or IFB0223) was collected from the frozen stock, streaked on Tryptic Soy Agar medium (TSA) (Oxoid, Basingstoke, UK) and incubated at 28 °C for 24 h. Subsequently, a single bacterial colony was picked from the TSA plate and used for inoculation of liquid M9 minimal medium with glucose as the primary carbon source that will be referred to as M9 throughout the manuscript[52]. These cultures were then incubated for 24 h at 28 °C with shaking at 130 rpm. Growth rates and biomass yields between the three biological replicates were comparable, confirming the reproducibility of the cultures. Subsequently, 100 µl of the overnight bacterial culture was transferred either to M9 (non-induced conditions, containing glucose) or M9 + PGA (induced conditions, in spite of glucose supplemented with 4 g L^{-1} PGA) (Agdia Biofords, Évry, France), and incubated for 48 h at 28 °C with 130 rpm shaking to mimic environmental conditions encountered at different stages of the infection process[19,53]. Basing on our former studies investigating the growth dynamics of *D. solani* IFB0099 and IFB0223, a 48-hour incubation period was selected as it captured the peak of bacterial growth and maximal enzyme activity, particularly in terms of the wild-type strains[39]. The selected incubation time allowed for optimal observation of the virulence factors activity and bacterial growth density under laboratory conditions mimicking the process of naturally-occurring infection. The overnight bacterial cultures were then centrifuged (7085 rcf for 10 min), and the resultant pellets were washed twice with 0.85% NaCl prior to suspending the cells by vigorous mixing in sterile physiological saline.

In terms of the methylome analysis, bacterial cells were grown under high nutrients availability in Luria Broth (LB) medium at 28 °C for 24 h with shaking at 200 rpm[5]. These growth conditions were selected to ensure optimal bacterial growth and allow for an insight into the global DNA methylation patterns of IFB0099 and IFB0223.

Detection of the methylated DNA motifs

During our former study, the genomes of *D. solani* IFB0099 and IFB0223 were sequenced on a Pacific Biosciences platform as a commercial service by BaseClear (The Netherlands)[5]. 118,344 and 102,248 PacBio reads were acquired for *D. solani* IFB0099 and IFB0223, respectively. Initially, bax.h5 files were converted to bam files using bax2bam version 0.0.9 (github.com/EichlerLab/bax2bam) on the Galaxy webserver[54]. Then, identification of the methylated motifs was performed using the smrtlink version 7.0.1.66975 command line tools (Pacific Biosciences). Specifically, the bam files were first mapped to the corresponding reference genome (GCF_000831935.2 for *D. solani* IFB0099 and GCF_003718335.1 for *D. solani* IFB0223) using pbalign version 0.4.1. The methylated sites and motifs were then detected in each genome using ipdSummary version 2.4 and motifMaker.

RNA isolation

Total RNA was extracted according to the manufacturer's instructions using a commercially available Bead-Beat Total RNA Mini kit (A&A Biotechnology, Gdansk, Poland) from the cells of *D. solani* IFB0099 and IFB0223, suspended in 0.85% NaCl post culturing either in M9 or M9 + PGA. The isolated RNA was purified and concentrated using Clean-Up RNA Concentrator and Total RNA Mini Concentrator (A&A Biotechnology, Gdansk, Poland) kits following the protocols provided by the producer.

The purity and concentration of RNA in each sample were evaluated spectrophotometrically using NanoDrop ND-1000 (Thermo Fisher Scientific, Minneapolis, USA). In addition, Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) was implemented to examine the concentration, integrity (RNA integrity number; RIN), and 23S/16S rRNA ratio in the RNA samples. Three biological replicates of the isolated RNA were included for each of the two *D. solani* strains across the two studied conditions.

Analysis and visualization of the RNA-Seq data

Samples of the isolated RNA from *D. solani* strains (minimum 5 µg of RNA; concentration 65 ng/µl) were sent to Genomed (Warsaw, Poland) for the sequencing service. There, rRNA was depleted using QIAseq FastSelect – 5S/16S/23S kit (Qiagen, Hilden, Germany). Afterwards, a strand-specific cDNA library was prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs Inc., Massachusetts, USA). The sequencing process was performed on a MiSeq sequencing platform (Illumina, San Diego, USA) to reach a coverage of ten million paired-end reads per sample. The sequencing depth was selected on the basis of the recommended number of reads for conducting differential expression study[55]. Three biological replicates were performed for each of the two strains across the two examined conditions. The RNA-Seq data obtained for *D. solani* strains of different levels of virulence are publicly available in the Sequence Read Archive repository at http://www.ncbi.nlm.nih.gov/bioproject/944547.

Raw RNA-Seq reads were stripped of adapter sequences during conversion of the raw reads to the FASTQ format. To achieve ten million pairs of reads per sample, four subsets of forward reads in addition to four subsets of reverse reads were generated for each biological replicate. Trimming of the reads was performed using Trimmomatic (version 0.39)[56] as follows: adapter removal was based on the file with the adapters sequences provided by the Genomed company, quality trimming was applied using a sliding window approach with a window size of five and a minimum average quality of 20 and a minimum length of 50 bases after trimming. After this step, the quality of the trimmed reads was checked with FASTQC[57]. Finally, the reads from each of the four subsets per replicate that passed the step of quality control have been combined into one dataset containing high quality reads of a single biological sample.

The trimmed RNA-Seq reads were mapped *ab initio* to the reference genome of *D. solani* type strain IPO 2222 (GCF_001644705.1) using Bowtie2 (version 11.2.0)[58]. The quantitative gene expression analysis involved identification of DEGs from RNA-Seq data by Expectation-Maximization (RSEM)[59], primarily using an absolute log2FoldChange > |1| and p adj < 0.05 as the selection criteria. However, for specific analyses, such as the heatmaps, GO enrichment analysis and the examination of genes encoding major virulence factors, DEGs were selected based on p adj < 0.05 without applying the Log2FoldChange threshold, to capture all statistically significant changes in gene expression levels. DEGs were further studied with the DESeq2 package (p adj < 0.05)[60] in R (RStudio version 2021.09.0). The computed diagnostic tests included comparison identifier MA and a Volcano plot. In addition, a two-dimensional chart of similarities between the samples from the analyzed PCA collections was generated for all the expressed genes. To visualize gene expression levels of the analyzed strains, a heatmap of the normalized expression values on the re-filtered DEGs in R (ggplot2 package) was generated. The expression values for the filtered genes were transformed to log2 scale in a way that minimizes differences between samples for rows with small counts, and which normalizes with respect to the library size ("regularized log" transformation). Finally, upregulated and downregulated genes under the induced (IFB0099 vs. IFB0223) and non-induced (IFB0099 vs. IFB0223) conditions were identified based on Log2FoldChange > |1| and p adj < 0.05. Then, the enrichment of GO terms was analyzed to unveil the overrepresented biological processes among the DEGs under the induced (IFB0099 vs. IFB0223) and non-induced (IFB0099 vs. IFB0223) conditions. The analysis was conducted using the TopGO package in R, with DEGs filtered according to an adjusted p adj < 0.05. Fisher's exact test was applied to evaluate the significance of enrichment within the "Biological Process" GO ontology. The results were visualized as dot plots, where the size of the dots represents the gene ratio, calculated as the proportion of DEGs associated with a given GO term relative to the total number of DEGs

in the analysis. For the analysis of differential expression of genes encoding major virulence factors in the case of IFB0099 (induced vs. non-induced conditions) and IFB0223 (induced vs. non-induced conditions), all DEGs were included, regardless of their statistical significance. For the *arcZ* gene, which has not been annotated in the reference genome of *D. solani* IPO 2222, its differential expression was analyzed manually by directly quantifying normalized read counts in the genomic region corresponding to *arcZ*, based on similarities to homologous sequences from the genomic annotations of other *Dickeya* strains. Finally, functional annotations of the DEGs were corrected basing on high quality gene annotations from the genome of *D. dadantii* 3937, which is deposited in the NCBI database under the accession number ASM14705v1. The assignment of functions to the identified proteins relied on data deposited in the UniProt database (Universal Protein Knowledgebase; accessed 09.2023).

Data availability

The raw RNA-Seq data obtained for *Dickeya solani* strains of different levels of virulence are publicly available in the Sequence Read Archive repository at http://www.ncbi.nlm.nih.gov/bioproject/944547. All other data generat ed or analyzed during this study are included in this published article (or in its supplementary information files).

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Author contributions

E. L., A. M-P and W. B-W. conceived and designed all experiments. W. B-W. cultured bacteria, isolated RNA and performed bioinformatic analyses on the RNA-Seq data. W. B-W, A. M., G. C. D. took part in the discussion on the selection of appropriate bioinformatics tools for visualization of the RNA-Seq data. W. B-W. visualized the collected data. G. C. D. performed methylation analysis. W. B-W, A. M-P., E. L., A. M., G. C. D. took part in discussion. W. B-W. wrote the first version of this manuscript. W. B-W, A. M-P., A. M., G. C. D., E. L . corrected the manuscript. Additionally, W. B-W., A. M-P., and E. L. addressed the reviewers' comments. The submitted version of the manuscript has been accepted by all the authors. E. L. acquired funding for this study and administered the corresponding project.

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Declarations

Competing interests
The authors declare no competing interests.

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